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<b>(54) Title:</b> MODULATORS OF PNEUMOCOCCAL ADHERENCE TO PULMONARY AND VASCULAR CELLS AND DIAGNOSTIC AND THERAPEUTIC APPLICATIONS  <b>(57) Abstract</b>  The present invention relates to compositions and methods for preventing pneumococcal infection. In particular, this invention relates to the identification of the minimum receptor targets of pneumococcal adherence to pulmonary and vascular endothelium, and to compositions and methods for preventing such adherence. In particular, the invention relates to the ability of one or more carbohydrate entities having the following motif or motifs: a disaccharide N-acetyl-D-galactosamine $\beta$ 1-3Gal motif, a disaccharide N-acetyl-D-galactosamine $\beta$ 1-4Gal motif, and an N-acetyl-D-glucosamine motif, effective to induce elution of adherent <i>S. pneumoniae</i> from host cells. In particular, a composition containing all three motifs can elute about 100 % of pneumococcal bacteria from lung epithelial cells, and from venous endothelial cells. In a particular embodiment, a pharmaceutical composition of the invention can be used to treat pneumococcal infections in which the host cells are lung epithelial cells. For use in blocking adherence to, or eluting adherent bacteria from, lung epithelial cells, the pharmaceutical composition is an aerosol formulation. The invention further provides formulations for parenteral administration for treating systemic blood-borne infections by preventing or reversing binding to venous endothelial cells. The invention further relates to methods and kits for determining the presence of pneumococci in a biological sample from a subject.		

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**MODULATORS OF PNEUMOCOCCAL ADHERENCE TO PULMONARY  
AND VASCULAR CELLS AND DIAGNOSTIC AND  
THERAPEUTIC APPLICATIONS**

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**FIELD OF THE INVENTION**

10 The present invention relates to compositions and methods  
for preventing pneumococcal infection. In particular,  
this invention relates to the identification of the  
targets of pneumococcal adherence to pulmonary and  
vascular endothelium, and to compositions and methods for  
preventing such adherence.

15 **BACKGROUND OF THE INVENTION**

Streptococcus pneumoniae is the most frequent cause of  
bacterial pneumonia in children of developing countries  
and accounts for up to 76% of cases in adults  
(Pennington, 1986, Am. Fam. Physician 33:153; Johnston,  
20 1991, Revs. Infect. Dis. 13 (Suppl. 6):S509; Musher,  
1992, Clin. Infect. Dis. 14:801). Pneumococci generally  
enter the host via the nasopharynx where they attach to  
epithelial cells and in some instances persist for  
several months (Anderson et al., 1981, Infect. Immun.  
25 32:311). In experimental models, progression to  
pneumonia results from spread of the pneumococci by  
aerosolization and conveyance from the nasopharynx down  
into the lower respiratory tract.

The early pneumonic lesion is characterized by fluid  
30 filled alveoli containing pneumococci, which are  
frequently seen to line the alveolar walls, a  
distribution suggestive of a specific interaction

promoting retention in the alveolar space (Wood, 1941, J. Exp. Med. 73:222). Pneumococci readily gain access to the blood circulation from the alveolar space, suggesting an aggressive capability to cross the vascular  
5 endothelial cells of the alveolar capillaries (Rake, 1936, J. Exp. Med. 63:191).

Bacterial adherence to eukaryotic cells commonly involves specific bacterial proteins (adhesions) which recognize host cell glycoconjugates. Adherence of pneumococci to  
10 human oral epithelium is inhibited in the presence of the disaccharide N-acetyl-glucosamine- $\beta$ 1-3-galactose (GlcNAc $\beta$ 1-3Gal) suggesting that this carbohydrate can serve as a receptor, perhaps relevant to nasopharyngeal carriage (Anderson et al., 1983, J. Exp. Med. 158:559).  
15 It remains unknown precisely which cell type supports pneumococcal adherence in the alveolar space. Pneumococci have been reported to bind to purified glycoconjugates containing terminal or internal GalNAc $\beta$ 1-4Gal, a structure prevalent in pulmonary secretions and  
20 lung tissue (Krivan et al., 1988, Proc. Natl. Acad. Sci. USA 85:6157). This structure differs significantly in stereochemistry from that proposed for the nasopharyngeal receptor.

The identity of pneumococcal adhesions capable of  
25 mediating attachment to the nasopharynx and lung is unknown. Consistent with the ability to readily cause experimental bacteremia, pneumococcal adherence to vascular endothelial cells has been shown to be dose-dependent, rapid and independent of capsular type (Geelen  
30 et al., 1993, Infect. Immun. 61:1538). Both cell-wall components and protein components contribute roughly equally to this association, but specific pneumococcal ligands remain to be identified (Tuomanen et al., 1985, J. Infect. Dis. 151:859).

The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

#### SUMMARY OF THE INVENTION

- 5 In a first aspect, the present invention is directed to a pharmaceutical composition comprising an amount of a carbohydrate containing a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal motif effective to induce elution of adherent Streptococcus pneumoniae from host cells.
- 10 The disaccharide may be linked to mannose. The carbohydrate component of the pharmaceutical composition of the invention can competitively inhibit the binding of pneumococci to host cells, particularly lung epithelial cells and vascular endothelial cells. Competitive
- 15 inhibition of pneumococcal binding, termed herein "adhesion," can prevent colonization of susceptible host cells by pneumococci, or elute adherent pneumococci from host cells.

- Thus, a particular advantage of the invention is that it
- 20 provides a treatment for pneumococci that is independent of antibiotics. More particularly, the present invention can be efficacious for treating antibiotic and multiple antibiotic resistant bacteria. Alternatively, the pharmaceutical compositions and methods of the present
- 25 invention can be used in conjunction with antibiotics to treat an infection suspected of or known to be a pneumococcal infection.

- In a more preferred embodiment, the pharmaceutical composition comprises an amount of a second carbohydrate
- 30 containing a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal motif effective to induce elution of adherent S. pneumoniae from host cells. This second group can also be linked to mannose.

A further discovery of the invention is that activated host cells exhibit yet another saccharide specificity for pneumococcal adhesion: N-acetyl-D-glucosamine (GlcNAc).

Accordingly, in another preferred embodiment, the

- 5 pharmaceutical composition comprises an amount of a third carbohydrate containing an N-acetyl-D-glucosamine motif effective to induce elution of adherent S. pneumoniae from host cells.

In the most preferred embodiment, the pharmaceutical

- 10 composition of the invention comprises an amount of each of the three carbohydrates, a carbohydrate containing a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal motif, a second carbohydrate containing a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal motif, and a third carbohydrate  
15 containing an N-acetyl-D-glucosamine motif, effective to induce elution of adherent S. pneumoniae from host cells. In particular, this composition can elute about 100% of pneumococcal bacteria from lung epithelial cells, and from venous endothelial cells.

- 20 The inventors herein have observed that additional carbohydrate groups can be included in a composition of the invention. For example, but not by way of limitation, a composition of the invention may further comprise an amount of one or more of mannose, N-acetyl-D-  
25 galactosamine, mannose-D-mannose, and methyl- $\alpha$ -D-mannopyranoside effective to inhibit binding of pneumococci to a host cell.

In a preferred aspect, the carbohydrates contain a multiplicity of pneumococcal binding motifs, i.e., the

- 30 carbohydrates are polyvalent or multivalent. In one embodiment, a carbohydrate is multivalent for one motif. In another embodiment, a carbohydrate contains more than one motif, and may be multivalent for each. Multivalence can be achieved using branching complex oligosaccharides;

alternatively, monovalent carbohydrates can be conjugated to a polymer carrier, such as a protein, cyclodextran, polylysine, polyacrylamide, agarose, SEPHAROSE, and the like. Preferably, 20 to 30 such motifs are attached to each polymer.

In a particular embodiment, a pharmaceutical composition of the invention can be used to treat pneumococcal infections in which the host cells are lung epithelial cells. For use in blocking adherence to, or eluting adherent bacteria from, lung epithelial cells, the pharmaceutical composition is an aerosol formulation, which formulation contains a dispersant. For example, the dispersant can be a surfactant. In one specific embodiment, the aerosol formulation is a dry powder aerosol formulation, in which the carbohydrate or carbohydrates are present in finely divided powder. In another embodiment, the aerosol formulation is a liquid aerosol formulation, which includes a pharmaceutically acceptable diluent. Possible diluents include, but are not limited to, sterile water, saline, buffered saline, and dextrose solution.

The present invention further extends to a pharmaceutical composition that can be used to treat pneumococcal infections that have spread into the blood, in which the host cells are vascular endothelial cells. Pneumococcal infection of blood follows pulmonary infection with significant frequency, and can lead to severe complications, such as but not limited to, sepsis, bacteremia, and meningitis. Thus, the invention relates to pharmaceutical compositions for injection into the blood, in particular for intravenous injection, to treat pneumococcal infections that have spread into the blood.

Accordingly, the invention relates to a method for preventing or treating an infection with S. pneumoniae

comprising administering to a subject believed to be in need of such treatment an amount of a carbohydrate containing mannose linked to a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal group effective to induce elution  
5 of adherent S. pneumoniae from host cells. In a preferred embodiment, the method further comprises administering an amount of a second carbohydrate containing mannose linked to a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal group effective to induce elution  
10 of adherent S. pneumoniae from host cells. In another preferred embodiment, the method further comprises administering an amount of a third carbohydrate containing N-acetyl-D-glucosamine effective to induce elution of adherent S. pneumoniae from host cells. In  
15 the most preferred embodiment, the method comprises administering an amount of each of the three carbohydrates, a carbohydrate containing mannose linked to a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal group, a second carbohydrate containing mannose linked to a  
20 disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal group, and a third carbohydrate containing N-acetyl-D-glucosamine, effective to induce elution of adherent S. pneumoniae from host cells.

As noted above, the invention particularly relates to  
25 treating infections of lung epithelial cells. Accordingly, a method for treating an infection with S. pneumoniae comprises atomizing and inhaling the carbohydrate or carbohydrates. Preferably, the atomization is with a nebulizer to create a sonicated  
30 mist that is readily inhaled.

The invention also particularly relates to treating infections of the blood. Accordingly, a method for treating a blood infection with S. pneumoniae comprises injecting the carbohydrate or carbohydrates  
35 intravenously.



In another aspect, the present invention relates to a method for detecting the presence of S. pneumoniae comprising detecting the binding of a carbohydrate containing mannose linked to a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal group to bacteria. In a further embodiment, detecting the presence of pneumococci further comprises detecting the binding of a second carbohydrate containing mannose linked to a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal group to bacteria. In another embodiment, the detecting the presence of pneumococci further comprises detecting the binding of a third carbohydrate containing N-acetyl-D-glucosamine to bacteria.

In the most preferred embodiment, the method for detecting the presence of S. pneumoniae comprises detecting the binding of all three carbohydrate to bacteria.

According to the invention, the carbohydrate or carbohydrates are detectably labeled; alternatively, the carbohydrate or carbohydrates may be attached to a solid phase support.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Adherence of pneumococci to primary rabbit pneumocytes. A) Adherence of encapsulated AII to mixed lung cells (○ -○) and purified Type II lung cells (■ - ■). B) Comparison of the binding of encapsulated AII (■ - ■) and isogenic, unencapsulated R6 pneumococci (○ - ○) to Type II lung cells. For both panels, adherent bacteria were enumerated by plating for cfu and by visualization by Gram stain. Values are the means  $\pm$  SD of six experiments. \* $p < 0.05$  versus mixed lung cells.

FIGURE 2. Dose-response curve (A) and time course (B) for adherence of pneumococci to human cultured Type II pneumocytes. For the dose-responsive curve, Type II pneumocytes were incubated with increasing concentration of AII (■ - ■), R6 (□ - □), D39 (○ - ○), 18C (▲ - ▲) or 9V (△ - △) for 30 min. For the time, course experiments type II pneumocytes were incubated with  $1 \times 10^7$  cfu/ml R6. Adherent bacteria were determined as the number of attached bacteria per 100 type II lung cells. Results are the means  $\pm$  SD for duplicate wells in at least six independent experiments.

FIGURE 3. Effect of glycolipids containing the sequences GalNAc $\beta$ 1-4Gal and GalNAc $\beta$ 3Gal on pneumococcal adherence to human cultured lung cells (A) and endothelial cells (B). R6 was incubated with GalNAc $\beta$ 1-4Gal-containing glycolipids [asialo-GM2 (• - •) or asialo-GHM1 (○ - ○)] or GalNAc $\beta$ 1-3Gal-containing glycolipids [forssman glycolipid (□ - □) or globoside (■ - ■)]. Adherent bacteria were determined as the number of attached bacteria per 100 lung cells or endothelial cells. Control adherence in the absence of sugar is indicated by the horizontal line (---). Results are the means  $\pm$  SD of duplicate well sin at least six independent experiments.

FIGURE 4. Antiadherence effect of globoside, asialo-GM2 and purified pneumococcal cell wall. A) R6 was incubated with either albumin buffer (---), globoside (■ - ■) or asialo-GM2 (• - •) or the combination of asialo-GM2 plus globoside (♦ - ♦); final concentration indicated was composed equally of the 2 sugars) and allowed to adhere to Type II lung cell monolayers. \*p<0.05 compared to asialo-GM2 or globoside alone. B) Monolayers were incubated (30 min., 37°C) with either pneumococcal cell wall (50  $\mu$ g/ml) or albumin buffer. R6 was incubated (15 min., room temperature) with wither

albumin buffer (control), globoside (2mM) or asialo-GM2 (2mM) and allowed to adhere to the monolayers for 30 min. at 37°C. Adherent bacteria were determined as the number of attached bacteria per 100 endothelial cells. Results are the means  $\pm$  SD of duplicate wells in at least six independent experiments. Wall is significantly less than control \* is significantly less than wall ( $p < 0.05$ ).

FIGURE 5. Minimal carbohydrate receptor structures involved in pneumococcal adherence to two separate classes of receptor (A, B) on cultured human Type II pneumocytes and endothelial cells. Bold typed, boxed areas represent active minimal receptor unit required for adherence of pneumococci to Type II pneumocytes or endothelial cells. Structures underlined are inactive in both cell types. Abbreviations as per Table 1, infra.

FIGURE 6. Effect of simple sugars on adherence of R6 resting and cytokine-stimulated human cultured type II pneumocytes (A) and vascular endothelial cells (B). R6 ( $10^7$  cfu/ml) was exposed to various simple sugars (15 min., room temperature) and then co-incubated with either resting (open bars) or cytokine-stimulated (solid bars) monolayers of cells for 30 min. Pneumococcal adherence to a designated sugar was defined as bacterial adherence to 100 host cells in the absence of sugar minus bacterial adherence to 100 host cells in the presence of sugar. Results are the means  $\pm$  SD for duplicate wells in at least six independent experiments. Glc = D-glucose; Gal = D-galactose; Fuc = L-fucose; GalNAc = N-acetyl-D-galactosamine; GlcNAc = N-acetyl-D-glucosamine; Man = D-mannose; NANA = sialic acid; Lac = lactose; and 2-ADGG = 2-acetamide-2-deoxy-3-O-B-D-galactopyranosyl-D-galactopyranose.

FIGURE 7. Pneumococcal glycoconjugate receptors on cytokine-stimulated monolayers. The number of

pneumococci adherent to the GalNAc $\beta$ 1-4Gal population was define by asialo-GM2 ( $\square$  -  $\square$ ) and those adherent to the GalNAc $\beta$ 1-3Gal population by globoside ( $\diamond$  -  $\diamond$ ). To define whether the carbohydrate specificities were contained in one or more receptors, the ability of two component mixtures of GlcNAc ( $\circ$  -  $\circ$ ) plus either globoside ( $\blacklozenge$  -  $\blacklozenge$ ) or asialo-GM2 ( $\blacksquare$  -  $\blacksquare$ ) to impair pneumococcal adherence to a greater degree than each sugar alone was assessed. The test compositions contained the single sugar or equal amounts of the two sugars tested. Control adherence to TNF $\alpha$ -stimulated monolayers is indicated by the hatched line (----). \* p < 0.05 compared to asialo-GM2 or GlcNAc alone. Pneumococcal adherence to each designated sugar was determined from the bacterial adherence to 100 host cells in the absence of sugar minus bacterial adherence to 100 host cells in the presence of sugar. Results shown are the means  $\pm$  SD for duplicate wells from at least six independent experiments.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for treating or preventing infection with S. pneumoniae. The present invention is based on the discovery that pneumococci recognize and bind to at least three different carbohydrate motifs, two of which were not recognized prior to this invention. In specific embodiments, the invention provides for inhibiting adhesion of pneumococci, or eluting adherent pneumococci, from lung epithelial cells.

The present inventors have discovered that a carbohydrate motif containing a GalNAc $\beta$ 1-3Gal group binds to pneumococci. This motif was previously not known to interact with pneumococcus. More importantly, the inventors have discovered that pneumococci have more than one carbohydrate specificity, and that inhibiting binding

to carbohydrates of only one such specificity cannot quantitatively elute the bacteria. Thus, using the preferred compositions and methods of the invention, quantitative elution (or prevention of adherence) can be  
5 achieved.

To provide a better understanding of the invention, definitions for certain terms are provided below; aspects of the invention -- carbohydrates; therapeutic compositions and methods; diagnostic compositions and  
10 methods; and Examples are also presented.

A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc.)  
15 when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by  
20 weight. It is also preferred that a composition, which is substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

The phrase "pharmaceutically acceptable" refers to  
25 molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term  
30 "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a

diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

- 10 As used herein, the term "host cell" refers to a cell capable of colonization by pneumococci, for example lung epithelial cells and venous endothelial cells.

As used herein, the term "pulmonary administration" refers to administration of a formulation of the invention into the lungs by inhalation.

As used herein, the term "inhalation" refers to intake of air to the alveoli. In specific examples, intake can occur by self-administration of a formulation of the invention while inhaling, or by administration via a respirator, e.g., to an patient on a respirator. The term "inhalation" used with respect to a formulation of the invention is synonymous with "pulmonary administration."

As used herein, the term "parenteral" refers to introduction of a carbohydrate that binds to pneumococcus into the body by other than the intestines, and in particular, intravenous (i.v.), intraarterial (i.a.), intraperitoneal (i.p.), intramuscular (i.m.), intraventricular, and subcutaneous (s.c.) routes. Preferably, the route is intravenous.

As used herein, the term "aerosol" refers to suspension in the air. In particular, aerosol refers to the

particalization of a formulation of the invention and its suspension in the air. According to the present invention, an aerosol formulation is a formulation comprising a carbohydrate that is aerosolized, i.e.,  
5 atomized and suspended in the air, for inhalation or pulmonary administration.

As used herein, the term "systemic" refers to a disease or disorder, or original site of injury distant to the lung or involving the entire body of the organism. The  
10 term "local" therefore is used herein with respect to the lung. Thus, a systemic infection is one in which S. pneumonia are found in the blood, and can lead to bacteremia and meningitis. A local infection is one in which the pneumococci have migrated only as far as the  
15 lung, and can lead to pneumonia.

The compositions of the invention, or the administration of a composition, can be used to protect or treat an animal subject from infection of S. pneumoniae. Thus, a composition of the invention can be used in birds, such  
20 as chickens, turkeys, and pets; in mammals, preferably a human, although the compositions of the invention are contemplated for use in other mammalian species, including but not limited to domesticated animals (canine and feline); farm animals (bovine, ovine, equine,  
25 caprine, porcine, and the like); rodents; and undomesticated animals.

Carbohydrate Motifs That Bind to Pneumococci

In its primary aspect, the present invention relates to the identification of the carbohydrate motifs that mediate adherence of pneumococci to host cells, in particular lung epithelial cells (Type II lung cells), and venous endothelial cells.

Thus, as used herein, the term "pneumococcal binding motif" refers to the structure of a carbohydrate that is recognized by the pneumococcal bacterial adhesion complex. It is well known that a carbohydrate (also termed oligosaccharide and saccharide) on a glycoprotein can be a highly complex structure formed by a set of monosaccharide (sugar) subunits arranged in a variety of linkages (see, e.g., Darnell et al., Molecular Cell Biology, Scientific American Books, 1986, pp. 957-964). The motif of the present invention is the minimum receptor unit, i.e., the minimum structure capable of binding with pneumococci, regardless of the "decoration" (additional saccharide subunits) found on the compound. Thus, the carbohydrate motif to which pneumococci bind can be located in a complex oligosaccharide, or may be the simplest possible oligosaccharide having the motif structure. Furthermore, a motif can be a derivative of the specific saccharide groups shown herein to mediate pneumococcal binding. A functional definition of a motif of the invention is that it can demonstrate pneumococcal binding and elution properties as described in the Examples herein.

As used herein, the term "carbohydrate" refers both to compounds containing saccharide subunits, and to such compounds associated or conjugated with a polymer as described herein.



- In a first aspect, the invention relates to identification of a carbohydrate motif to which pneumococci bind that has not previously been known to mediate pneumococci binding. This motif contains a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal group (GalNAc $\beta$ 1-3Gal). This motif may be, but need not be, linked to a mannose. Examples of carbohydrates that contain this motif include, but are not limited to, forssman glycolipid, globoside, etc.
- 10 In a further aspect, the inventors have discovered that quantitative or nearly quantitative elution of adherent pneumococci from host cells can be achieved by contacting the bacteria with the GalNAc $\beta$ 1-3Gal motif and a second motif, to which pneumococci were previously known to
- 15 adhere. This second motif is a carbohydrate containing a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal group (GalNAc $\beta$ 1-4Gal). This motif may be, but need not be, linked to a mannose. Examples of carbohydrates that contain this second motif include, but are not limited
- 20 to, asialo-GM1, asialo-GM2, etc. As shown in a specific example, infra, quantitative elution of pneumococci from unstimulated lung epithelial cells (Type II lung cells) and venous endothelial cells can be achieved by contacting these cells with a composition containing
- 25 carbohydrates having both motifs.

- In a further embodiment, the inventors herein have discovered that stimulation of the host cells to which pneumococci bind with inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), etc.,
- 30 induces a third previously unknown carbohydrate specificity: N-acetyl-D-glucosamine (GlcNAc). This sugar specificity is associated with the GalNAc $\beta$ 1-3Gal receptor population. Thus, a composition comprising a carbohydrate with the GalNAc $\beta$ 1-3Gal motif and the GlcNAc
- 35 motif (both motifs can be found on the same

oligosaccharide, or on two different oligosaccharide) can be used to prevent adherence of pneumococci to, or elute adherent pneumococci from, host cells.

- In the most preferred embodiment, a composition
- 5 comprising carbohydrates that represent all three motifs can be used. Such a composition is particularly desirable to treat an early stage, ongoing infection, in which the immune system has been activated and inflammatory mediators have been released.
- 10 The invention contemplates including additional carbohydrate moieties that have been found to interfere with binding or adherence of pneumococci with host cells. Examples of such carbohydrates include, but are not limited to, mannose, N-acetyl-D-galactosamine, mannose-D-
- 15 mannose, and methyl- $\alpha$ -D-mannopyranoside.

- The present invention further contemplates use of multivalent carbohydrates or carbohydrate-containing structures to increase the potency of the drug. In one embodiment, one motif is found in multiple copies on a
- 20 compound for use in the invention. In another embodiment, more than one motif is found in single or multiple copies on a compound for use in the invention. Multivalent carbohydrates can be prepared by preparing a branching complex carbohydrate, which conceptually
- 25 resembles a tree or brush in which each branch or bristle contains a pneumococcal binding motif. Alternatively, monovalent carbohydrates can be associated covalently or non-covalently with a polymer (e.g., Langer et al., International Patent Publication No. WO 94/03184,
- 30 published February 17, 1994, which is specifically incorporated herein by reference). Suitable polymers include, but are not limited to, a protein, polylysine, dextran, a glycosaminoglycan, cyclodextrin, agarose, SEPHAROSE, and polyacrylamide.

Carbohydrates with pneumococcal binding motifs according to the invention can be obtained from any source. For example, such carbohydrates can be obtained from commercial sources. Alternatively, the carbohydrates can  
5 be prepared synthetically, using known chemical or enzymatic processes. Glycosyltransferase enzymes for synthesis of a carbohydrate (i.e., saccharide) that contains a pneumococcal binding motif can be prepared as described in International Patent Publication No. WO  
10 93/13198 by Roth (published July 8, 1993), which is incorporated herein by reference. Glycosyltransferase-catalyzed preparation of saccharide compositions has also been described (Roth, U.S. Patent No. 5,180,674, issued January 19, 1993, and International Patent Publication  
15 No. 91/16449, published October 31, 1991), as has an apparatus for preparing such compositions (Roth, U.S. Patent No. 5,288,637, issued February 22, 1994) (each of these references is specifically incorporated herein by reference in its entirety).

Therapeutic Compositions and Methods

The present invention contemplates formulations comprising a carbohydrate containing a pneumococcal binding motif as described above for pulmonary or  
5 parenteral, especially i.v., administration for the prevention and treatment of pneumococcal infection, and resulting disease conditions including, but not limited to, bacteremia, meningitis, and pneumonia. Hereinafter, for convenience sake, the term "carbohydrate" in the  
10 singular or plural form should be interpreted to mean a carbohydrate containing a pneumococcal binding motif, unless another meaning is specifically provided.

Accordingly, the present invention provides pharmaceutical compositions comprising one or more  
15 carbohydrates containing a pneumococcal binding motif and a pharmaceutically acceptable carrier or excipient, as defined above. For the treatment of a nascent pneumococcal infection or a systemic infection, a therapeutic composition of the invention can be  
20 administered by inhalation, to prevent colonization of lung epithelial cells leading to infection, or to elute adherent bacteria from lung epithelial cells. Pulmonary administration is an effective mode of administration for the bloodstream as well, since drugs pass from the  
25 alveoli into the capillaries readily. Moreover since systemic pneumococcal infections generally begin with colonization of lung epithelial cells, administration of a therapeutic agent via the lungs is a rational route to treat the infection. Similarly, it is also possible for  
30 a drug administer parenterally, e.g., i.v., to cross from the capillaries to the alveoli. Accordingly, the present invention contemplates parenteral administration of a carbohydrate or carbohydrates, in particular i.v. administration, for treatment of both systemic and lung  
35 (local) pneumococcal infections.

Accordingly, a wide variety of devices that are designed for the delivery of pharmaceutical compositions and therapeutic formulations to the respiratory tract or parenterally can be used in this aspect of the invention.

5 The preferred route of pulmonary administration of the present invention is in the aerosol or inhaled form. The carbohydrate or carbohydrates of the present invention, combined with a dispersing agent, or dispersant, can be administered in an aerosol formulation  
10 as a dry powder or in a solution or suspension with a diluent. However, as mentioned above, the composition of the invention can also be administered parenterally.

As used herein, the term "dispersant" refers to a agent that assists aerosolization of the carbohydrate or  
15 absorption of the carbohydrate in lung tissue, or both. Preferably the dispersant is pharmaceutically acceptable. For example, surfactant that are generally used in the art to reduce surface induced aggregation of the carbohydrates caused by atomization of the solution  
20 forming the liquid aerosol may be used. Non-limiting examples of such surfactant are surfactant such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitan fatty acid esters. Amounts of surfactant used will vary, being generally within the  
25 range or 0.001 and 4% by weight of the formulation. Suitable surfactant are well known in the art, and can be selected on the basis of desired properties, depending on the specific formulation, concentration of carbohydrate or carbohydrates, diluent (in a liquid formulation) or  
30 form of powder (in a dry powder formulation), etc.

Moreover, depending on the choice of the carbohydrate or carbohydrates (e.g., disaccharide or complex oligosaccharide), the desired therapeutic effect, the quality of the lung tissue (e.g., diseased or healthy  
35 lungs), and numerous other factors, the liquid or dry

formulations can comprise additional components, as discussed further below.

The liquid aerosol formulations contain the carbohydrate or carbohydrates and a dispersing agent in a  
5 physiologically acceptable diluent. The dry powder aerosol formulations of the present invention consist of a finely divided solid form of the carbohydrate or carbohydrates and a dispersing agent. The parenteral formulations contain the carbohydrate or carbohydrates in  
10 a suitable carrier for injection.

With either the liquid or dry powder aerosol formulation, the formulation must be aerosolized. That is, it must be broken down into liquid or solid particles in order to ensure that the aerosolized dose actually reaches the  
15 alveoli. In general the mass median dynamic diameter will be 5 micrometers or less in order to ensure that the drug particles reach the lung alveoli (Wearley, L.L., 1991, Crit. Rev. in Ther. Drug Carrier Systems 8:333). The term "aerosol particle" is used herein to describe  
20 the liquid or solid particle suitable for pulmonary administration, i.e., that will reach the alveoli. Other considerations such as construction of the delivery device, additional components in the formulation and particle characteristics are important. These aspects of  
25 pulmonary administration of a drug are well known in the art, and manipulation of formulations, aerosolization means and construction of a delivery device require at most routine experimentation by one of ordinary skill in the art.

30 With regard to construction of the delivery device, any form of aerosolization known in the art, including but not limited to nebulization, atomization or pump aerosolization of a liquid formulation, and aerosolization of a dry powder formulation, can be used

in the practice of the invention. Often, the aerosolization of a liquid or a dry powder formulation will require a propellant. The propellant may be any propellant generally used in the art. Specific non-  
5 limiting examples of such useful propellants are a chlorofluorocarbon, a hydrofluorocarbon, a hydrochlorofluorocarbon, or a hydrocarbon, including trifluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-  
10 tetrafluoroethane, or combinations thereof.

In a particular aspect of the invention, the device for aerosolization is a metered dose inhaler. A metered dose inhaler provides a specific dosage when administered, rather than a variable dose depending on administration.  
15 Such a metered dose inhaler can be used with either a liquid or a dry powder aerosol formulation. Metered dose inhalers are well known in the art.

Once the carbohydrate or carbohydrates reach the lung by inhalation, a number of formulation-dependent factors  
20 effect the binding activity. It will be appreciated that in treating a systemic pneumococcal infection, that requires circulatory levels of the carbohydrate or carbohydrates, such factors as aerosol particle size, aerosol particle shape, the presence or absence of lung  
25 disease or emboli that may affect the absorption of the carbohydrates, pH of the lungs or the pharmaceutical carrier, etc. For each of the formulations described herein, certain lubricators, absorption enhancers, stabilizers or suspending agents may be appropriate. The  
30 choice of these additional agents will vary depending on the goal. It will be appreciated that in instances where local delivery of the carbohydrate or carbohydrates is desired or sought, such variables as absorption enhancement will be less critical.

In a further embodiment, an aerosol or parenteral formulation of the present invention can include other active ingredients in addition to the carbohydrate or carbohydrates. In a preferred embodiment, such active ingredients are those used for the treatment of lung disorders. For example, such additional active ingredients include, but are not limited to, bronchodilators, antihistamines, epinephrine, and the like, which are useful in the treatment of pulmonary conditions. In a preferred embodiment, the additional active ingredient can be an antibiotic, e.g., for the treatment of pneumonia. In a preferred embodiment, the antibiotic is pentamidine.

While in a preferred aspect the compositions of the present invention are administered in conjunction with antibiotics, it is a particular advantage of the present invention that it provides a strategy for treating antibiotic resistant pneumococcus. This is an important consideration in view of the recent increase in antibiotic resistance among virulent bacteria, a phenomenon that is of grave public health concern.

In general, the carbohydrate or carbohydrates of the present invention, are introduced into the subject in the aerosol or parenteral form in an amount between 0.01 mg per kg body weight of the mammal up to about 100 mg per kg body weight of said mammal. In a specific embodiment, the dosage is dosage per day. One of ordinary skill in the art can readily determine a volume or weight of aerosol corresponding to this dosage based on the concentration of carbohydrate or carbohydrates in an aerosol or parenteral formulation of the invention; alternatively, one can prepare an aerosol formulation which with the appropriate dosage of carbohydrate or carbohydrates in the volume to be administered, as is readily appreciated by one of ordinary skill in the art.



It is also clear that the dosage will be higher in the case of inhalation therapy for a systemic pneumococcal infection, and may be lower for treating an infection of the lung only, since the local concentration of

5 carbohydrate or carbohydrates in the lung will be much higher with pulmonary administration. It is an advantage of the present invention that administration of a carbohydrate or carbohydrates directly to the lung allows targeted drug delivery, thus limiting both cost and

10 unwanted side effects.

The formulation may be administered in a single dose or in multiple doses depending on the severity of the infection or need for prophylaxis. For example, when administered to a subject on a respirator as protection

15 against pneumococcal infection (a common consequence of long-term treatment on a respirator), less of the composition may be effective. If an infection commences, more of the composition can be provided to facilitate elution of adherent bacteria as well as prevent adhesion

20 and colonization by resident bacteria. It will be appreciated by one of skill in the art the exact amount of prophylactic or therapeutic formulation to be used will depend on the stage and severity of the disease, the physical condition of the subject, and a number of other

25 factors, which can be readily determined by the skilled physician (see, e.g., Langer et al., International Patent Publication No. WO 9403184, published February 17, 1994, which is specifically incorporated herein by reference).

Systems of aerosol delivery, such as the pressurized

30 metered dose inhaler and the dry powder inhaler are disclosed in Newman, S.P., Aerosols and the Lung, Clarke, S.W. and Davia, D. editors, pp. 197-22 and can be used in connection with the present invention.

Liquid Aerosol Formulations

The present invention provides liquid aerosol formulations and dosage forms for use in treating subjects suffering from or in danger of acquiring a  
5 pneumococcal infection. In general such dosage forms contain one or more carbohydrates in a pharmaceutically acceptable diluent. Pharmaceutically acceptable diluents include but are not limited to sterile water, saline, buffered saline, dextrose solution, and the like. In a  
10 specific embodiment, a diluent that may be used in the present invention or the pharmaceutical formulation of the present invention is phosphate buffered saline, or a buffered saline solution generally between the pH 7.0-8.0 range, or water.

15 The liquid aerosol formulation of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, surfactant and excipients.

The liquid aerosol formulations of the present invention  
20 will typically be used with a nebulizer. The nebulizer can be either compressed air driven or ultrasonic. Any nebulizer known in the art can be used in conjunction with the present invention such as but not limited to: Ultravent, Mallinckrodt, Inc. (St. Louis, MO); the Acorn  
25 II nebulizer (Marquest Medical Products, Englewood CO). Other nebulizers useful in conjunction with the present invention are described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627  
30 issued January 13, 1987.

The formulation may include a carrier. The carrier is a macromolecule which is soluble in the circulatory system and which is physiologically acceptable where

physiological acceptance means that those of skill in the art would accept injection of said carrier into a patient as part of a therapeutic regime. The carrier preferably is relatively stable in the circulatory system with an  
5 acceptable plasma half life for clearance. Such macromolecules include but are not limited to Soya lecithin, oleic acid and sorbetan trioleate, with sorbitan trioleate preferred.

The formulations of the present embodiment may also  
10 include other agents useful for stabilization or for the regulation of osmotic pressure. Examples of the agents include but are not limited to salts, such as sodium chloride, or potassium chloride, and carbohydrates, such as glucose, galactose or mannose, and the like.

15                   Aerosol Dry Powder Formulations

It is also contemplated that the present pharmaceutical formulation will be used as a dry powder inhaler formulation comprising a finely divided powder form of the carbohydrate or carbohydrates and a dispersant. The  
20 form of the carbohydrate will generally be a lyophilized powder. Lyophilized forms of carbohydrates can be obtained through standard techniques.

In another embodiment, the dry powder formulation will comprise a finely divided dry powder containing one or  
25 more carbohydrates, a dispersing agent and also a bulking agent. Bulking agents useful in conjunction with the present formulation include such agents as lactose, sorbitol, sucrose, or mannitol, in amounts that facilitate the dispersal of the powder from the device.

Diagnosis of a Pneumococcal Infection

- The carbohydrates identified herein as binding to pneumococci are valuable reagents for the diagnosis of an infection with S. pneumoniae. Presently, diagnosis of
- 5 infection with a Gram positive bacterium, including pneumococci, is difficult. Usually, diagnosis depends on a combination of symptomatology, an X-ray pattern consistent with pneumonia, and in vitro culturing of biological samples obtained from a subject suspected of
- 10 having a pneumococcal infection. Such samples are typically obtained from sputum, bronchial lavage, extraction of lung fluid with a needle, and blood (which detects the presence of pneumococci that have infected blood).
- 15 According to the invention, the presence of S. pneumoniae in a sample from a subject suspected of having an infection with S. pneumoniae can be detected by detecting binding of a carbohydrate as described above to bacteria in or from the sample. As noted above, the
- 20 invention provides the unexpected discovery that pneumococci adhere to carbohydrates containing mannose linked to a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-3Gal group. Accordingly, detecting binding of this carbohydrate motif to bacteria is indicative that the
- 25 bacteria are pneumococci. Furthermore, detection of binding to the GalNAc $\beta$ 1-3Gal and either or both the GalNAc $\beta$ 1-4Gal and the GlcNAc groups with bacteria can provide more definitive information about the identity of the bacteria as pneumococci.
- 30 According to the invention, the detection of binding of one or more of the aforementioned carbohydrate motifs can be detected independently, i.e., binding to each carbohydrate motif is detected separately. Separate detection can be accomplished by performing separate

assays for each type of carbohydrate, or by using distinct labels on each carbohydrate for which binding is to be assayed. Alternatively, binding to all two or all three motifs can be detected simultaneously.

- 5 Detection of binding of carbohydrates with bacteria according to the invention can be assayed in a manner analogous to an immunoassay. In the present case, the carbohydrate acts as one binding partner, and the bacterium acts as another binding partner.
- 10 In one embodiment, a quantity of one or more carbohydrates that bind with pneumococci can be prepared and optionally labeled, such as with an enzyme, a compound that fluoresces and/or a radioactive element, and may then be introduced into a tissue or fluid sample
- 15 of a mammal believed to be infected with pneumococci. After the labeled material has had an opportunity to react with sites within the tissue, the resulting mass may be examined by known techniques, which may vary with the nature of the label attached. Preferably if such in
- 20 vivo detection is desired, the radio-label is technetium-99 (<sup>99</sup>Tc), or a magnetic shift reagent label such as gadolinium or manganese, is used as a label.

The presence of pneumococci in animals can be ascertained in general by immunological procedures, which utilize one

25 or more carbohydrate binding partner to pneumococci, in which one component is labeled with a detectable label. In a preferred aspect, the presence of pneumococci is ascertained in an assay that involves a carbohydrate labeled with a detectable label. In a specific aspect,

30 the detectable label may be the binding partner of another reagent, e.g., biotin (which binds with avidin or streptavidin), or an antigen for an antibody. The procedures may be summarized by the following equations wherein the asterisk indicates that the molecular species

is labeled, and "C" in this instance stands for the carbohydrate or carbohydrates, and "B" stands for the bacterium:

- A.  $C^* + B + C = C^*B$   
5 B.  $C_1 + C_2^* + B = C_1BC_2^*$

- These general procedures and their application are all familiar to those skilled in the art and are presented herein as illustrative and not restrictive of procedures that may be utilized within the scope of the present  
10 invention. An immunoassay procedure analogous to the "competitive" procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752. An immunoassay analogous to procedure B, the "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and 4,016,043.
- 15 A further alternate diagnostic procedure employs multiple labeled compounds in a single solution for simultaneous radio-assay. In this procedure, analogous to the procedure disclosed in U.S. Patent No. 4,762,028 to Olson, a composition may be prepared with two or more  
20 carbohydrate molecules in a coordinated compound having the formula: radioisotope-chelator-carbohydrate molecule.

- The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce  
25 when exposed to ultraviolet light, and others.

- Suitable radioactive elements may be selected from the group consisting of  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{36}\text{Cl}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , and  $^{186}\text{Re}$ . In the instance where a radioactive label, such as one of the isotopes listed  
30 above, is used, known currently available counting procedures may be utilized to detect or quantitate the amount of label, and thus the amount of material bound.

In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, thermometric, amperometric or gasometric techniques known in the art. The enzyme may be conjugated to the carbohydrate, by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase,  $\beta$ -glucuronidase,  $\beta$ -D-glucosidase,  $\beta$ -D-galactosidase, urease, glucose oxidase plus peroxidase, hexokinase plus GDPase, RNase, glucose oxidase plus alkaline phosphatase, NAD oxidoreductase plus luciferase, phosphofructokinase plus phosphoenol pyruvate carboxylase, aspartate aminotransferase plus phosphoenol pyruvate decarboxylase, and alkaline phosphatase. U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternative labeling material and methods.

A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine and auramine.

Various assay formats are also contemplated by the present invention for detecting the presence, and if desired, the amount, of pneumococcal bacteria in a sample. For example, a direct "sandwich"-type ELISA can be performed, in which one carbohydrate is attached to the solid phase support, and a labeled carbohydrate (which may share the same motif as the first, or which may have one of the other motifs) is used to detect binding of bacteria to the solid phase carbohydrate.

The present invention includes assay systems that may be prepared in the form of test kits for the quantitative

analysis of the extent of the presence of pneumococcal bacteria. The system or test kit may comprise a labeled component, e.g., prepared by one of the radioactive and/or enzymatic techniques discussed herein, coupling a  
5 label to a carbohydrate binding partner of pneumococci. Alternatively, a test kit of the invention may comprise such a carbohydrate associated with or capable of being associated with a solid phase support, including but not limited to, a microtiter plate, chromatography beads  
10 (SEPHAROSE and the like), and cells (for rosetting experiments).

In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of  
15 pneumococci. Such kits can also be used to determine the amount of pneumococci in a sample. In accordance with the testing techniques discussed above, one class of such kits will contain at means for detecting binding of a carbohydrate as described herein and a pneumococcus, and  
20 may include directions, depending upon the method selected. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

For example, a first assay format contemplates a bound carbohydrate to which is added the biological sample  
25 suspected of containing pneumococci, followed by a labeled carbohydrate (or anti-pneumococci antibody). The resulting substrate is then washed, after which detection proceeds by the measurement of the amount of label specifically retained on the solid phase.

30 All of the protocols disclosed herein may be applied to the qualitative and quantitative determination of pneumococci and to the concomitant diagnosis and surveillance of S. pneumoniae infection.



The present invention will be better understood from a review of the following illustrative description presenting the details of the constructs and procedures that were followed in its development and validation.

5        EXAMPLE: RECEPTOR SPECIFICITY OF ADHERENCE OF  
         STREPTOCOCCUS PNEUMONIAE

The adherence of S. pneumoniae to human type II pneumocytes and endothelial cells is critical to the pathogenesis of pneumococcal pneumonia and bacteremia.

10 This example demonstrates that the preferred target cell to which pneumococci adhere in the lung is the type II lung cell and reports an in vitro adherence assay to determine the molecular details of this interaction. Pneumococcal receptors on cultured human lung cells (LC)  
15 and endothelial cells (EC) appeared to be glycoproteins, since treatment of the monolayers with tunicamycin (an inhibitor of glycosylation) significantly impaired bacterial adherence.

Inhibition of adherence to LC and EC occurred following  
20 incubation with several carbohydrates including, GalNAc, mannose and GalNAc $\beta$ 1-4Gal- and GalNAc $\beta$ 1-3Gal-containing glycoconjugates. Pneumococci could bind directly to these immobilized sugars and their addition to adherent pneumococci could elute the bacteria from LC and EC.  
25 Combinations of glycoconjugates indicated that two independent classes of pneumococcal receptor existed on both cell types. These were defined by the minimal receptor units GalNAc $\beta$ 1-4Gal and GalNAc $\beta$ 1-3Gal which participate in pneumococcal cell wall and protein-  
30 dependent mechanisms of adherence, respectively.

Materials and Methods

Bacterial strains and cultivation conditions. *S. pneumoniae* strains of serotype 2 (AII and D39) and serotypes 18C, 9V, and the unencapsulated strain R6 (isogenic with D39 and AII) were grown on trypticase soy agar (Difco, Detroit, MI) containing 3% sheep blood (Micropure Medical Inc., Stillwater, MI) for 18 h at 37°C. Bacteria were grown at 37°C. Bacteria were harvested from the plate into 1 ml Dulbecco's phosphate buffered saline (DPBS; Whittaker Bioproducts, Watersville, MD) and labelled with fluorescein isothiocyanate (FITC 1 mg/ml; Sigma Chemical Company, St. Louis, MO) dissolved in a buffer containing 0.05 M sodium carbonate and 0.1 M sodium chloride as previously described (Geelen et al., 1993, Infect. Immun. 61:1538). The bacteria were washed twice by centrifugation (3,000 rpm, 3 min), resuspended in 1 ml albumin buffer (Geelen et al., supra) and diluted to between  $10^5$  and  $10^{10}$  cfu/ml. For some experiments, R6 pneumococci were grown in defined medium with either choline or ethanolamine as the aminoalcohol (Tuomanen et al., 1985, J. Infect. Dis. 151:859).

Pneumococcal cell wall preparation. Purified whole cell wall from R6 was prepared as previously described (Geelen et al., supra). Briefly, logarithmically growing pneumococci were heat-killed and crude cell wall extracted in 5% sodium dodecyl sulfate (SDS; 100°C, 30 min). SDS was removed by washing and the cell wall treated with DNase, RNase and trypsin. After reprecipitation in boiling SDS, the walls were washed, lyophilized, and stored at room temperature. For use in bioassays, pneumococcal cell wall suspensions were homogenized by sonification with a Branson Sonifier (model 2200; Branson Ultrasonic Corp., Danbury, CT) and

resuspended to a final concentration of 50-100 mg/ml in albumin buffer.

These concentrations were chosen as they had previously been shown to produce maximum inhibition of pneumococcal adherence to human endothelial cells (Geelen et al., supra).

Rabbit mixed and type II lung cell preparations. Rabbit mixed and type II lung cells were prepared as previously described (Dobbs and Mason, 1979, J. Clin. Invest. 63:378). Briefly, lungs were minced and digested with collagenase, elastase and DNase for 60 min at 37°C. Large pieces were removed using a gauze filter and cells were pelleted and washed twice. The resulting mixed lung cells were resuspended in 20 ml of calcium-containing buffer with 0.5% albumin to a density of  $10^6$ /ml. To purify alveolar type II cells from the mixed lung cell suspension, the mixture was layered on an albumin gradient of 10 ml 16.5g% over 10 ml 35g% and centrifuged at 1200 rpm for 20 min at 4°C. The top 26 ml of the gradient was discarded and cells in the next 12 ml were harvested, washed and adjusted to a concentration of  $10^6$ /ml.

Cell viability was >90% as assessed by trypan blue exclusion and >80% of the cells contained osmiophilic lamellar bodies typical of type II lung cells when examined by electron microscopy.

Human type II pneumocytes and vascular endothelial cells. The human type II lung cell line A549 (LC; American type culture collection) was added to tissue culture flasks coated with gelatin (0.2%) and cultured in Nutrient mixture F12 Ham medium (Sigma) supplemented with 10% fetal calf serum (Sigma). Primary cultures of human umbilical vein endothelial cells (EC; passage 1,

Clonetics Corp., San Diego, CA) were added to tissue culture flasks coated with fibronectin (50  $\mu$ g/ml) and grown in Medium 199 (Sigma) supplemented as previously described (Krivan et al., 1988, Proc. Natl. Acad. Sci. USA 85:6157). In order to determine the influence of glycosylation of proteins on pneumococcal adherence to LC and EC, some monolayers were prepared from cells grown for 96 h at 37°C in the presence of tunicamycin (0.55  $\mu$ g/ml; Sigma) (Dobbs and Mason 1979, J. Clin. Invest. 63:378). At confluence the cells were prepared for subculture with trypsin-0.05% EDTA (Sigma). For adherence assays, cells were transferred to Terasaki 60-well culture dishes (Robbins Scientific, Sunnyvale, CA) coated with fibronectin (50 mg/ml) and cultured for another 24 to 48 h to form a confluent monolayer.

Prior to the adherence assay, culture fluid was removed by washing the monolayers twice with medium 199 (Sigma).

Adherence of bacteria to primary rabbit mixed lung cells.

To study adherence of pneumococci to primary rabbit mixed lung cells and type II pneumocytes, AII or R6 ( $10^3 - 10^9$  cfu/ml) were added to  $10^4$  lung cells in a 1 ml volume and incubated under rotation for 30 min at 37°C. Lung cells were then separated from non-adherent bacteria by washing six times by centrifugation (270 g, 5 min) and resuspension in medium 199. Bacteria adherent to the final cell pellet were then enumerated both by plating and visualization by Gram stain and the baseline established from six independent experiments. These quantitation techniques were chosen to ensure that adherence reflected viable bacteria without the potential complications of a bacterial surface derivatized with a detection label. Subsequent comparison of the adherence of killed bacteria and fluorescein labelled bacteria to the adherence of viable cells indicated no significant differences (see below).

Adherence of pneumococci to cultured human LC and EC.

Adherence of pneumococci to LC and EC was investigated using the protocol previously described for cultures of human vein endothelial cells (Geelen et al., 1993, 5 Infect. Immun. 61:1538). FITC-labelled bacteria ( $10^5$  to  $10^8$  cfu/ml; 10  $\mu$ l/well) were incubated with cell monolayers for up to 150 min at 37°C. Pneumococcal adherence was also assessed at 4°C.

Nonadherent bacteria were removed by washing the 10 monolayers 5 times with medium 199 and the cells were then fixed in 2.5% glutaraldehyde. Adherent pneumococci were counted visually with an inverted microscope (Diaphot-TMD; Nikon Inc., Melville, N.Y.) equipped for fluorescence with an IF DM-510 filter. Adherence was 15 expressed as the number of attached bacteria per 100 eukaryotic cells counted in a 40x field. Values for two wells were averaged and each experiment was performed on at least six separate occasions. Inter-and-intra assay coefficients of variation of the adherence assay were 20 found to be 18% and 15% respectively.

To test the ability of pneumococcal cell wall to inhibit adherence, cultured LC and EC were incubated with cell wall (5  $\mu$ l; 50 or 100  $\mu$ g/ml) for 30 min at 37°C before the addition of labelled bacteria. For experiments to 25 define the ability of carbohydrates to inhibit adherence, FITC-labelled R6 ( $2 \times 10^7$  cfu/ml) were pre-incubated for 15 min at room temperature (Tuomanen et al., 1988, J. Exp. Med. 168:267) with final concentrations of one of the following as listed in Table 1: monosaccharides at 30 between 1 and 50 mM, glycoconjugates at 0.002 - 2 mM (Sigma), or albumin buffer without glucose (control).

Bacteria were centrifuged (3,000 rpm, 3 min) to remove the sugar, resuspended to  $1 \times 10^7$  cfu/ml in albumin buffer and added to the adherence assay. Adherence was assessed

after 30 min at 37°C of co-incubation of R6 and the LC or EC monolayers. Results were expressed as the number of bacteria attaching to a designated sugar. This value is derived from the difference between adherence in the absence or sugar (control wells) and the presence of sugar. For example, if 100 bacteria adhere in control wells and 60 bacteria adhere when sugar A is added, then it is calculated that 40 bacteria (40%) adhere to cells in a manner dependent on sugar A.

- 10 Adherence of pneumococci to immobilized glycoconjugates. Solid-phase binding assays were carried out according to Lee and colleagues (Lee et al., 1994, Mol. Microbiol. 11:705). Stock solutions of the monosaccharides (100 mM) and complex carbohydrates (2 mM) were prepared in 10%  
15 chloroform in methanol (v/v). Dilutions of the monosaccharides (12-50 mM) and complex carbohydrates (0.004-0.4 mM) were made in methanol.

Terasaki 60-well culture dishes (Robbins Scientific, Sunnyvale, CA) were coated with 10 µl per well of either  
20 the monosaccharides or glycoconjugates in methanol, or methanol as control, and allowed to evaporate to dryness in at 4°C. The wells of the microtitre plate were then blocked by incubation (1 h, 37°C) with 5% (w/v) bovine serum albumin (BSA; Sigma) in phosphate buffered saline  
25 (PBS), pH 7.4.

The excess BSA was decanted and the wells washed once with 0.05% BSA in PBS. Fluorescein labelled pneumococci ( $10^7$  cfu/ml) were allowed to adhere to the plates for 30 min at 37°C and adherence quantified visually using an  
30 inverted fluorescence microscope.

Statistics. Differences between groups were tested by the Wilcoxon signed ranks test. All results are expressed as means and standard deviations of at least six experiments.

5

### Results

#### Interaction of Pneumococci with Lung Cells

Adherence of pneumococci to primary lung cells. Adherence of encapsulated AII pneumococci to mixed rabbit lung cells was concentration dependent and saturable (Figure 1A).  
10 Adherence to purified type II lung cells was consistently 10-100 fold greater at each inoculum than to mixed lung cells suggesting the type II lung cell was the preferred target. At the maximum level of adherence,  $10^5$  cfu were associated with  $10^6$  type II cells indicating an adherence  
15 index of ~10 pneumococci per cell. Adherence to purified type II cells was equivalent for encapsulated AII and isogenic unencapsulated R6 suggesting that the capsule did not affect pneumococcal attachment (Figure 1B).

Adherence of R6, AII, D39, 9V and 18C to human type II lung cell line. A model of pneumococcal adherence to type  
20 II pneumocytes was developed using the A549 type II LC line. Encapsulated strains AII, D39, 9V and 18C and the unencapsulated R6 adhered in a dose-dependent fashion (Figure 2A). A threshold dose of approximately  $10^6$  cfu/ml  
25 was required to detect attachment. With higher concentrations, adherence increased in a linear fashion to  $\geq 10$  bacteria per cell, a value similar to the maximum for primary type II cells. The attachment of pneumococci to LC reached a plateau within 45 min (Figure 2B). To  
30 investigate whether active metabolism was required for adherence, live and heat-killed R6 (10 min, 60°C) were compared in the adherence assay. At an input concentration of  $10^7$  cfu/ml, the number of attached bacteria per 100 LC

was  $296 \pm 10$  and  $273 \pm 14$  ( $p > 0.05$ ) for live and heat-killed R6, respectively.

Role of pneumococcal cell wall in adherence of R6 to human LC. Pretreatment of type II LC monolayers (30 min,  $37^{\circ}\text{C}$ ) with purified pneumococcal cell wall ( $50 \mu\text{g/ml}$ ) significantly ( $p < 0.05$ ) decreased the adherence of  $10^5$ ,  $10^6$  and  $10^7$  cfu/ml of R6 to LC by  $39 \pm 12\%$ ,  $47 \pm 17\%$  and  $57 \pm 7\%$ , respectively. Similar results were obtained using  $100 \mu\text{g/ml}$  of pneumococcal cell wall (data not shown). To directly assess the role of the phosphorylcholine moiety present in the cell wall teichoic acid in adherence, pneumococci were cultured in medium containing ethanolamine, which biosynthetically substitutes the aminoalcohol in the teichoic acid. At an input concentration of  $10^7$  cfu/ml, the number of R6 adherent to 100 LC was  $273 \pm 14$  and  $294 \pm 14$  ( $p > 0.05$ ) for pneumococci grown with choline and ethanolamine, respectively.

#### Receptor Specificity for Pneumococci on LC and EC

Effect of tunicamycin on pneumococcal adherence. Tunicamycin inhibits the glycosylation of cell surface glycoproteins by blocking the first step in the biosynthetic pathway of the oligosaccharide portion of asparagine-linked high-mannose- and complex-type glycoproteins (Hranitzky et al., 1985, Infect. Immun. 49:336). Adherence of pneumococci to monolayers treated with tunicamycin was significantly ( $p < 0.05$ ) impaired compared with untreated monolayers. At an input concentration of  $10^7$  cfu/ml of R6, the number of pneumococci adherent to 100 cells was  $67 \pm 9$  and  $240 \pm 15$  for EC cultured in the presence and absence of tunicamycin. Similar results were obtained for LC.

The carbohydrate specificity of the receptor(s) for pneumococcus on human LC and EC. The minimal receptor unit



was defined using competition assays in which simple and complex carbohydrates were used to interfere with S. pneumoniae strain R6 adherence to human LC and EC. Comparison of the ability of monosaccharides to inhibit adherence indicated that N-acetyl-D-galactosamine (GalNAc) and D-mannose played a role in >40% of the pneumococcal adherence to EC and LC; D-galactose was an additional specificity on EC only (Table 1). Since the pattern of competitive inhibition by monosaccharides does not necessarily predict the structure of a receptor (Lee et al., 1994, Mol. Microbiol. 11:705), the ability of a library of glycoconjugates containing mannose or N-acetyl-D-galactosamine to inhibit adherence was tested. The two mannose disaccharides mannose-D-mannose and methyl D-mannoside were effective receptor analogs for R6 adherence to both LC and EC (Table 1). Substitution of the terminal D-mannoside with D-galactoside led to a complete loss of activity of the molecule (Table 1).

TABLE 1 Antiadherence Activity of Monosaccharides and Glycoconjugates

Monosaccharide/Glycoconjugate	Carbohydrate Specificity#	Number of Pneumococci Adherent to Designated Sugar on Host Cell of total)*	
		Lung Cell	Endothelial Cells
Control		270 ± 10	240 ± 15
<b>Monosaccharides (50 mM)</b>			
D-galactose	Gal	10 ± 2 (4%)	112 ± 6 (47%)
D-glucose	Glc	5 ± 4 (2%)	11 ± 8 (5%)
L-fucose		8 ± 3 (3%)	19 ± 15 (8%)
Sialic Acid		6 ± 4 (2%)	13 ± 6 (5%)
D-mannose		142 ± 11 (53%)	110 ± 6 (46%)
N-acetyl-D-galactosamine	GalNAc	137 ± 7 (51%)	108 ± 9 (55%)
N-acetyl-D-glucosamine	GlcNAc	5 ± 2 (2%)	18 ± 6 (8%)
<b>Glycoconjugates (2mM)</b>			
Mannose-D-mannose	$\alpha$ -D-man[1-3]D-man	121 ± 9 (45%)	146 ± 4 (61%)
Methyl- $\alpha$ -D-mannopyranoside		137 ± 17 (51%)	100 ± 6 (63%)
Methyl- $\alpha$ -D-galactopyranoside		18 ± 4 (7%)	49 ± 11 (20%)
2-ADGG	GalB1-3GalNAc	6 ± 3 (2%)	11 ± 3 (5%)
Lactose	GalB1-4Glc	11 ± 3 (4%)	66 ± 11 (28%)
Lactosylceramide	GalB1-4GlcB1-1Cer	5 ± 2 (2%)	7 ± 5 (3%)
Asialo-GM1	GalB1-3GalNAcB1-4GalB1-4GlcB1-1Cer	100 ± 13 (37%)	70 ± 9 (29%)
Asialo-GM2	GalNAcB1-4GalB1-4GlcB1-1Cer	140 ± 9 (52%)	140 ± 4 (58%)
Monosialo-GM1	GalB1-3GalNAcB1-4(NeuAc2-3)GalB1-4GlcB1-1Cer	42 ± 8 (16%)	4 ± 2 (2%)
Forsman Glycolipid	GalNAc1-3GalNAcB1-3Gal1-4GalB1-4GlcB1-1Cer	130 ± 7 (51%)	70 ± 12 (29%)
Globoside	GalNAcB1-3Gal1-4GalB1-4GlcB1-1Cer	140 ± 9 (52%)	110 ± 8 (47%)

#Gal = galactose, Glc = glucose, GalNAc = N-acetyl-D-galactosamine, GlcNAc = N-acetyl-D-glucosamine, 2-ADGG = 2-acetamido-2-deoxy-3-O-8-D-galactopyranosyl-D-galactopyranose, Man-D-Man = 3-O-A-D-mannopyranosyl-D-mannopyranose, and Cer = ceramide. \*Number of pneumococci adherent to sugar = bacterial adherence to 100 host cells in absence of sugar - bacterial adherence to 100 host cells in presence of sugar. Values given are means ± SD of six independent experiments.

Of the complex GalNAc-containing carbohydrates tested, two were found to be highly effective targets of adherence on both LC and EC: asialo-GM2 and globoside (Table 1). The lactosylceramide (GalNAc $\beta$ 1-4Glc1-1Cer) group representing the common core of both of these glycoconjugates was inactive at all concentrations investigated (0.003 - 2mM). This suggested that receptor activity was associated with the presence of GalNAc linked to Gal by either a  $\beta$ 1,4 linkage (asialo-GM2) or a  $\beta$ 1,3 linkage (globoside). Reversal of the positions of GalNAc and Gal, as in the disaccharide Gal $\beta$ 1-3GalNAc (2-ADGG) rendered the carbohydrate inactive.

A variety of GalNAc- $\beta$ 1,4-Gal-containing sugars were compared to determine how substitutions affected the potency of the  $\beta$ 1,4 linkage active in asialo GM-2. Addition of a terminal Gal to create asialo-GM-1 decreased the ability to serve as a receptor by ~10-fold for LC (half maximal inhibition occurred at  $10^2\mu\text{M}$  for asialo GM-2 and at  $10^3\mu\text{M}$  for asialo GM-1) and >100 fold for EC (half maximal inhibition was not reached at  $>10^3\mu\text{M}$  for asialo GM-1) (Figures 3A and 3B). The small effect of asialo GM-1 on LC was virtually eliminated by substitution with sialic acid (monosialo-GM-1) (Table 1).

A similar analysis for the effect of substitutions on the activity of the  $\beta$ 1-3 linkage produced different results for LC and EC. Addition of a terminal GalNAc to globoside (forssman glycolipid) decreased receptor activity on EC 25 fold (Figure 3B). In contrast, forssman glycolipid showed a substantially increased activity over globoside for LC in that the concentration range over which a reduction in R6 adherence could be detected was greater, although the same maximal inhibition of R6 adherence occurred (~52%) (Figure 3A).

To investigate whether the carbohydrate specificities identified in Table 1 and Figure 3 were contained in one or multiple receptors, combinations of sugars were tested looking for additive effects indicative of two independent receptor specificities. Two component mixtures were prepared containing concentrations which separately produced maximal inhibitory effects on R6 adherence. The results of these studies are shown in Table 2. Mannose was not found to be additive with any other sugar or glycoconjugate suggesting it was a component of each class of receptors. A combination of asialo-GM1 plus asialo-GM2 or forssman glycolipid plus globoside was not additive in either cell type. A significant additive effect, virtually eliminating pneumococcal adherence, was seen with a combination of asialo-GM2 and globoside for both EC and LC (Figure 4A). These results suggested that the B1,4 and B1,3 linked GalNAc-Gal were distinct receptors. This was further supported by the ability of purified pneumococcal cell wall to further decrease adherence in the presence of globoside from ~50% to 20% of control values while having no demonstrable additive effect to that of asialo-GM2 (Figure 4B).

TABLE 2 Effect of Combinations of Sugars on Pneumococcal Adherence to Cultured Human Lung and Endothelial Cells

Monosaccharide/ Glycoconjugate#	Number of Pneumococci Adherent to Designated Sugar on Host Cell ‡	
	Lung Cells	Endothelial Cells
Control	270 ± 10	240 ± 15
<u>Monosaccharides</u> (50 mM)		
GalNAc	137 ± 7	108 ± 9
Gal	10 ± 2	112 ± 6
D-Mannose	142 ± 11	110 ± 6
GalNAc + Gal	NT	120 ± 11
GalNAc + Man	141 ± 8	115 ± 8
Gal + Man	NT	188 ± 9
<u>Glycoconjugates</u> (2 Mm)		
Asialo-GM1	100 ± 13	70 ± 9
Asialo-GM2	140 ± 9	140 ± 4
Globoside	140 ± 9	110 ± 8
Forssman	130 ± 7	70 ± 12
Man-man	121 ± 9	146 ± 4
Asialo-GM2 + asialo-GM1	139 ± 11	142 ± 7
Globoside + forssman	134 ± 6	108 ± 10
Asialo-GM2 + globoside	64 ± 9*	40 ± 8*
Man-man + asialo-GM2	142 ± 5	147 ± 6
Man-man + globoside	143 ± 8	150 ± 4

GalNAc = N-acetyl-D-galactosamine, Gal = galactose, Man = mannose. NT = not tested.

#R6 incubated (15 min., room temp.) with sugar, centrifuged and co-incubated with monolayers (30 min. at 37°C). Final concentration indicated was composed of single sugar or equally of the 2 sugars tested.  
\*p<0.05 compared to single sugar alone.

‡ Bacterial adherence to 100 cells in absence of sugar - bacterial adherence to 100 cells in presence of sugar. Values given in the table are means ± SD of 6 experiments.

Receptor analogs with sufficient binding affinity can effectively compete with the host cell receptor so as to cause elution of bacteria. GalNAc or D-mannose (50 mM) eluted  $65 \pm 10\%$  and  $64 \pm 8\%$ , respectively, of the  
5 adherent bacteria on LC within 60 min. Similar results for GalNAc ( $58 \pm 9\%$ ) and D-mannose ( $68 \pm 7\%$ ) were obtained with EC. More effective elution of adherent pneumococci occurred following incubation of LC or EC with asialo-GM2 ( $80\mu\text{M}$ ;  $75 \pm 8\%$  and  $70 \pm 7\%$ , respectively)  
10 or globoside ( $80\mu\text{M}$ ;  $79 \pm 10\%$  and  $63 \pm 5\%$ , respectively). Virtually all bacteria ( $>95\%$ ) were eluted from either cell type by a combination of  $80\mu\text{M}$  each of globoside and asialo-GM2.

Direct adherence of pneumococci to immobilized  
15 carbohydrates. Consistent with the receptor analog activity demonstrated in adherence assays using eukaryotic cells, pneumococci were able to adhere directly to immobilized Gal, GalNAc, GlcNAc, mannose, asialo-GM1, asialo-GM2, forssman glycolipid and globoside  
20 (Table 3).

Pneumococcal adherence was greatest to asialo-GM2 and asialo-GM1, followed at 10-fold less to forssman glycolipid or globoside. Adherence to simple sugars was approximately 100-fold less than to glycoconjugates.

TABLE 3 Direct Adherence of Pneumococci to Immobilized Carbohydrates

Monosaccharide/ Disaccharide	Glycoconjugate		Adherent Bacteria#		Adherent Bacteria#		
	50 mM	25 mM	12.5 mM		0.4 mM	0.07 mM	0.04 mM
Gal	79 ± 6* 7	55 ± 5*	36 ±	Asialo-GM1	558 ± 21* 10*	406 ± 19*	86 ±
GalNAC	103 ± 10* 9	65 ± 8*	38 ±	Asialo-GM2	601 ± 18* 11*	439 ± 24*	94 ±
Glc	35 ± 4 5	30 ± 6	32 ±	Globoside	302 ± 27* 9*	101 ± 13*	64 ±
GlcNAC	95 ± 4* 4	66 ± 2*	36 ±	Forssman Glycolipid	118 ± 20* ± 8*	94 ± 7*	76
Fucose	36 ± 12 8	33 ± 4	35 ±	Lactosylceramide	45 ± 8 40 ± 6	43 ± 4	
Mannose	99 ± 11* 4	75 ± 8*	38 ± 9				
Sialic Acid	38 ± 9 4	41 ± 3	40 ±				
Lactose	61 ± 2* 7	39 ± 5	39 ±				
2-ADGG	37 ± 9 4	41 ± 8	40 ±				

Gal = galactose, GalNAC = N-acetyl-d-galactosamine, Glc = Glucose, GlcNAc = N-acetyl-d-glucosamine and 2-ADGG = 2-acetamido-2-deoxy-3-O-B-D-galactopyranosyl-d-galactopyranose.

#Pneumococci (10<sup>7</sup> cfu/ml) were incubated with immobilized simple sugars (12.5 - 50 mM) or glycoconjugates (0.04 - 0.4 mM) for 30 min. at 37°C and the number of adherent bacteria in 100x microscope field was enumerated as usual using an inverted fluorescence microscope. Values given in the table represent means ± SD of 6 experiments with each experiment being the mean value of 3 replicate wells.

\*Significantly (p<0.05) greater than control adherence. Control (absence of sugar) = 39 ± 8 pneumococci.

Discussion

Pneumococcal pneumonia is characterized by the appearance of bacteria closely lining the alveolar epithelium. Based on the ability of pneumococci to adhere to mixed lung cells (predominately type I alveolar cells) and purified type II cells, it appears that S. pneumoniae adheres preferentially to the type II lung cell (LC). This suggested that the A549 type II LC line could serve as a valid model for pneumococcal attachment in the diseased lung. Using an in vitro adherence assay to a type II LC line several aspects of the mechanism of this interaction have been elucidated. Pneumococcal binding to cultures of LC was dose-dependent, rapid, did not require the bacteria to be metabolically active and was unaffected by the presence of several types of capsule. These findings are entirely in accord with previous studies of pneumococcal attachment to human vein endothelial cells (Geelen et al., 1993, Infect. Immun. 61:1538), suggesting similarities in the process of adherence to the two cells types. The interaction between pneumococci and endothelial cells has been shown to involve two separate mechanisms: ~60% of the bacterial adherence is mediated by cell-wall dependent mechanisms with the remaining 40% being protein mediated (Geelen et al., 1993, Infect. Immun. 61:1538). This study demonstrates that, like EC, approximately half of the pneumococcal attachment to LC involves a cell wall-dependent mechanism. In neither case is the phosphorylcholine determinant on the pneumococcal teichoic acid critical to adherence.

Like many classical mucosal pathogens, pneumococci can recognize and attach to glycoconjugates (Andersson et al., 1983, J. Exp. Med. 158:559; Krivan et al., 1988, Proc. Natl. Acad. Sci. USA 85:6157). The finding that inhibition of LC and EC cell surface glycosylation by



tunicamycin significantly decreased pneumococcal adherence indicates the potential relevance of carbohydrate binding in the context of eukaryotic cells. This Example discloses the carbohydrate specificity of the glycoconjugate receptors on cultured LC and EC that interact with pneumococci. Several lines of evidence indicate that both LC and EC possess two separate classes of glycoconjugate-containing receptors whose minimal units consist of mannose linked to the disaccharides GalNAC $\beta$ 1-4Gal or GalNAC $\beta$ 1-3Gal. Carbohydrates that mimicked the receptors (as indicated by the ability to block adherence when preincubated with pneumococcus) included mannose, N-acetyl-D-galactosamine (GalNAC), galactose (Gal; EC only), asialo-GM1, asialo-GM2, forssman glycolipid and globoside. Pneumococci were also able to bind directly to these carbohydrates when immobilized on microtitre plates, but much less so or not at all and glycoconjugates tested.

Most importantly, the incubation of cultured LC and EC bearing adherent bacteria with GalNAC, mannose, asialo-GM2 or globoside caused elution of the adherent pneumococci. A combination of asialo-GM2 and globoside produced significantly greater antiadherence effects for both LC and EC than when either glycoconjugate was used alone. This suggested that these glycoconjugates defined independent receptors. In addition, the antiadherence effects of purified pneumococcal cell wall were not additive with asialo-GM2 but were additive with globoside in both cell types. Pneumococcal adherence to EC and LC involves bacterial cell wall-dependent and independent (protein) mechanisms. The results of this study suggest that the asialo-GM2 specificity recognizes the cell wall component and globoside, the protein component.

The two pneumococcal receptor specificities consisted of the same saccharide substituents linked in distinctly

different stereochemical configurations: GalNAc linked in either a  $\beta$ 1-4 or a  $\beta$ 1-3 linkage to Gal (Figure 5). A combination of two  $\beta$ 1-4 GalNAc-Gal linked sugars, asialo-GM1 and asialo-GM2 possessed no greater antiadherence activity than either used singly. This was also true of the two  $\beta$ 1-3 GalNAc-Gal linked sugars, forssman glycolipid and globoside. However, combinations across the two classes eliminated adherence. These two pairs of glycoconjugates therefore appear to define two separate classes of receptor (Figure 5, A and B). The minimal receptor unit for each backbone was further defined by testing core structures and substituted variants for antiadherence effects. The core lactosylceramide (Gal $\beta$ 1-4Glc-1-1Cer) and the inverted disaccharide 2-ADGG (Gal $\beta$ 1-3GalNAc) were inactive in both cell types, and Gal was only minimally active in EC. No permissive substitutions to asialo-GM2 were found (addition of a terminal Gal or internal sialic acid decreased activity), thus defining the minimal active unit for this receptor as GalNAc $\beta$ 1-4Gal (Figure 5A). Similar analysis suggested the GalNAc $\beta$ 1-3Gal structure as the second minimal receptor unit with a more permissive substitution pattern for the LC as compared to the EC (Figure 5B). Addition of a terminal GalNAc8,1a1,4-linked to GalNAc $\beta$ 1-3Gal increased the receptor activity for LC but decreased its efficacy in EC. This indicates that subtle differences exist between the receptor structures on these two cell types.

The ability of bacteria to recognize both internal and terminal carbohydrate sequences has been previously described (Krivan et al., 1988, Proc. Natl. Acad. Sci. USA 85:6157; Karlssen, 1989, Annu. Rev. Biochem. 58:309). Mannose is frequently present in the core of N-linked saccharides of glycoproteins, a finding which could explain the predicted presence of mannose in each receptor identified in this study. Mannose was not additive when used in combination with either GalNAc,

- GalNAc $\beta$ 1-4Gal- or GalNAc $\beta$ 1-3Gal-containing sugars, suggesting that it is present in both pneumococcal receptors on LC and EC. A large number of mannose-binding bacteria have been described (Gbarah et al., 1993, Infect. Immun. 61:1687). Since mannose has not been detected in the glycolipids of higher animals, it has been suggested that the bacteria which bind mannose may selectively recognize glycoproteins (Karlssen, 1989, Annu. Rev. Biochem. 58:309).
- 10 In agreement with Krivan and colleagues (Krivan et al., 1988, Proc. Natl. Acad. Sci. USA 85:6157), this study demonstrates that pneumococci can specifically attach to immobilized glycoconjugates containing the sequence GalNAc $\beta$ 1-4Gal, including asialo-GM1 and asialo-GM2 and
- 15 that sialylation of this sequence reduces binding. This study further indicates that this structure functions as a minimal receptor sequence on LC and EC for a pneumococcal cell wall determinant. Of these glycolipids, asialo-GM1 has been shown to be present on
- 20 human lung epithelial cells (Krivan et al., supra). Sialylated glycoproteins containing GalNAc $\beta$ 1-4Gal sequences are also present in a number of secretions and are able to bind bacteria when the sialic acid is cleaved off (Krivan et al., supra). It has been suggested that
- 25 neuraminidase activity associated with influenza virus may increase the adherence of pneumococcus to the respiratory tract in vivo (Plotowski, 1986, Am. Rev. Respir. Dis. 134:1040). Studies by Andersson and colleagues (Andersson et al., 1983, J. Exp. Med. 158:559)
- 30 using human nasopharyngeal cells identified a pneumococcal receptor possessing a minimum sequence of GlcNAc $\beta$ 1-3Gal.

This structure is present in the saccharide chains of many glycolipids and glycoproteins including blood group

35 antigens (Hakamori, 1981, Semin. Hematol. 18:39). In our

studies the monosaccharide GlcNAc possessed no antiadherence activity for EC and LC, although pneumococci were able to bind directly to GlcNAc immobilized on microtitre plates. Thus, this receptor  
5 specificity may play a role in the nasopharynx that is distinct from that in the lung.

This study identified a new receptor specificity for pneumococcal adherence: #GalNAc $\beta$ 1-3Gal, which was involved in ~50% of the adherence to both LC and EC and  
10 appeared to interact with structures independent of the cell wall on the pneumococcal surface. Krivan and colleagues (Krivan et al., 1988, Proc. Natl. Acad. Sci. USA 85:6157) failed to detect binding of pneumococci to surfaces coated with the  $\beta$ 1-3 sugars, including forssman  
15 glycolipid or globoside.

This discrepancy could result from the requirement for a 10-fold greater concentration of globoside than asialo-GM2 to detect binding. Since both GalNAc $\beta$ 1-3Gal and GalNAc $\beta$ 1-4Gal containing glycoconjugates were required to  
20 elute pneumococci from LC and EC, both receptors appear to be physiologically relevant. This underlines the importance of using living cells to determine the relative contributions of host glycoconjugate receptors to bacterial adherence. Furthermore, therapeutic  
25 application of saccharides to promote clearance of pneumococci from the lower respiratory tract preferably interrupts interactions with both  $\beta$ 1-4 and  $\beta$ 1-3 GalNAc-Gal receptors.

EXAMPLE: SPECIFICITY OF ADHERENCE TO ACTIVATED CELLS

30 As discussed above, during the course of pneumococcal infection, pneumococci interact sequentially with nasopharyngeal cells, followed by lung epithelial cells (leading to pneumonia) or endothelial cells (leading to

- bacteremia, sepsis, and meningitis). Resting epithelial and endothelial cells bear two classes of glycoproteins bearing saccharide groups specific for binding pneumococci: GalNAc $\beta$ 1-3Gal and GalNAc $\beta$ 1-4Gal.
- 5 Pneumococcal infection is associated with the release of inflammatory cytokines, including tumor necrosis factor (TNF) and interleukin-1 (IL-1). Cytokine activation of human vein endothelial cells and type II lung cells resulted in greatly enhanced pneumococcal adherence. In
- 10 a TNF or IL-1 activated cell, a new saccharide specificity appears: N-acetyl-D-glucosamine (GlcNAc) (see Figure 6). GlcNAc-binding activity is associated with the GalNAc $\beta$ 1-3Gal glycoprotein cell population (see Figure 7).
- 15 The contribution of carbohydrate recognition to pneumococcal adherence to cytokine-stimulated EC and LC was tested using competition assays performed in the presence of monosaccharides (1-50 mM) or glycoconjugates (0.003-2 mM). Bacteria were pre-incubated for 15 min at
- 20 room temperature with final concentrations of the sugars as stated, centrifuged (3,000 rpm, 3 min) to remove unbound sugar, resuspended to  $1 \times 10^7$  cfu/ml in albumin buffer and added to the adherence assay. Comparison of the ability of single sugars (Tuomanen et al., J. Exp.
- 25 Med. 168:267), to inhibit adherence indicated that while GalNAc and D-mannose were effective in resting cells, GlcNAc became effective in activated cells (Figure 4).
- Resting EC and LC express two classes of pneumococcal receptors containing GalNAc $\beta$ 1-4Gal or GalNAc $\beta$ 1-3Gal on a
- 30 mannose core. These two receptor specificities are best defined by the inhibitory activities of the glycoconjugate asialo-GM2 for the  $\beta$ 1-4 receptor and globoside for the  $\beta$ 1-3 receptor. Cytokine stimulation of EC and LC resulted in a greater number of both of these

resting cell receptors, and the appearance of the new GlcNAc specificity. This latter specificity was independent of the GalNAc $\beta$ 1-4Gal specificity since the inhibitors were additive; GlcNAc was related to the

5 GalNAc $\beta$ 1-3Gal receptor population as these inhibitors were not additive (Figure 5). If the effects of the sugars were non-additive then they were considered to occupy the same or linked receptors; if additive then they were interpreted to be present in separate

10 receptors.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all

15 respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various references are cited throughout this

20 specification, each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

- 1 1. A pharmaceutical composition comprising an amount of  
2 a carbohydrate containing a disaccharide N-acetyl-D-  
3 galactosamine  $\beta$ 1-3Gal motif effective to induce elution  
4 of adherent Streptococcus pneumoniae from host cells, and  
5 a pharmaceutically acceptable carrier.
- 1 2. The pharmaceutical composition of Claim 1, further  
2 comprising an amount of a second carbohydrate containing  
3 a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal motif  
4 effective to induce elution of adherent S. pneumoniae  
5 from host cells.
- 1 3. The pharmaceutical composition of Claim 1, further  
2 comprising an amount of a third carbohydrate containing  
3 an N-acetyl-D-glucosamine motif effective to induce  
4 elution of adherent S. pneumoniae from host cells.
- 1 4. The pharmaceutical composition of Claim 2, further  
2 comprising an amount of a third carbohydrate containing  
3 an N-acetyl-D-glucosamine motif effective to induce  
4 elution of adherent S. pneumoniae from host cells.
- 1 5. The pharmaceutical composition of Claim 1, 2, 3, or  
2 4 in which the carbohydrate is selected from the group  
3 consisting of forssman glycolipid and globoside.
- 1 6. The pharmaceutical composition of Claim 2 or 4 in  
2 which the second carbohydrate is selected from the group  
3 consisting of asialo-GM1 and asialo-GM2.
- 1 7. The pharmaceutical composition of Claim 3 or 4 in  
2 which the third carbohydrate is N-acetyl-D-glucosamine.

1 8. The pharmaceutical composition of Claim 1, 2, 3 or 4  
2 further comprising an amount of a carbohydrate selected  
3 from the group consisting of mannose, N-acetyl-galactose,  
4 mannose-D-mannose, and methyl- $\alpha$ -D-mannopyranoside  
5 effective to inhibit binding of S. pneumoniae to host  
6 cells.

1 9. The pharmaceutical composition according to claim 1,  
2 2, 3 or 4 in which the carbohydrate or carbohydrates are  
3 multivalent.

1 10. The pharmaceutical composition of Claim 1, 2, 3, or  
2 4, in which the pharmaceutical composition is an aerosol  
3 formulation, which formulation contains a dispersant.

1 11. The pharmaceutical composition of Claim 10 in which  
2 the dispersant is a surfactant.

1 12. The pharmaceutical composition of Claim 10, which is  
2 a dry powder aerosol formulation, in which the  
3 carbohydrate or carbohydrates are present in finely  
4 divided powder.

1 13. The pharmaceutical composition of Claim 12 which  
2 further comprises a bulking agent.

1 14. The pharmaceutical composition of Claim 10, which is  
2 a liquid aerosol formulation further comprising a  
3 pharmaceutically acceptable diluent.

1 15. The pharmaceutical composition of Claim 14 in which  
2 the diluent is selected from the group consisting of  
3 sterile water, saline, buffered saline, and dextrose  
4 solution.



1 16. The pharmaceutical composition of Claim 1, 2, 3, or  
2 4 in which the host cell is selected from the group  
3 consisting of lung epithelial cells and vascular  
4 endothelial cells.

1 17. A method for preventing or treating an infection  
2 with S. pneumoniae comprising administering to a subject  
3 believed to be in need of such treatment an amount of a  
4 carbohydrate containing a disaccharide N-acetyl-D-  
5 galactosamine  $\beta$ 1-3Gal motif effective to induce elution  
6 of adherent S. pneumoniae from host cells.

1 18. The method according to Claim 17, further comprising  
2 administering an amount of a second carbohydrate  
3 containing a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-  
4 4Gal motif effective to induce elution of adherent S.  
5 pneumoniae from host cells.

1 19. The method according to Claim 17, further comprising  
2 administering an amount of a third carbohydrate  
3 containing an N-acetyl-D-glucosamine motif effective to  
4 induce elution of adherent S. pneumoniae from host cells.

1 20. The method according to Claim 17, further comprising  
2 administering an amount of a third carbohydrate  
3 containing an N-acetyl-D-glucosamine motif effective to  
4 induce elution of adherent S. pneumoniae from host cells.

1 21. The method according to Claim 17, 18, 19, or 20 in  
2 which the carbohydrate is selected from the group  
3 consisting of forssman glycolipid and globoside.

1 22. The method according to Claim 18 or 20 in which the  
2 second carbohydrate is selected from the group consisting  
3 of asialo-GM1 and asialo-GM2.

1 23. The method according to Claim 19 or 20 in which the  
2 third carbohydrate is N-acetyl-D-glucosamine.

1 24. The method according to Claim 17, 18, 19, or 20  
2 further comprising administering an amount of a  
3 carbohydrate selected from the group consisting of  
4 mannose, N-acetyl-galactose, mannose-D-mannose, and  
5 methyl- $\alpha$ -D-mannopyranoside effective to inhibit binding  
6 of S. pneumoniae to host cells.

1 25. The method according to Claim 17, 18, 19, or 20 in  
2 which the carbohydrate or carbohydrates are multivalent.

1 26. The method according to Claim 17, 18, 19, or 20 in  
2 which the administering comprises atomizing and inhaling  
3 the carbohydrate or carbohydrates.

1 27. The method according to Claim 26 in which the  
2 atomizing is nebulizing.

1 28. The method according to Claim 17, 18, 19, or 20 in  
2 which the administering comprises injecting the  
3 carbohydrate or carbohydrates intravenously.

1 29. A method for detecting the presence of S. pneumoniae  
2 in a sample from a subject comprising detecting the  
3 binding of a carbohydrate containing a disaccharide N-  
4 acetyl-D-galactosamine  $\beta$ 1-3Gal motif to bacteria.

1 30. The method according to Claim 29, further comprising  
2 detecting the binding of a second carbohydrate containing  
3 a disaccharide N-acetyl-D-galactosamine  $\beta$ 1-4Gal motif to  
4 bacteria.

1 31. The method according to Claim 29, further comprising  
2 detecting the binding of a third carbohydrate containing  
3 an N-acetyl-D-glucosamine motif to bacteria.

1 32. The method according to Claim 30, further comprising  
2 detecting the binding of a third carbohydrate containing  
3 an N-acetyl-D-glucosamine motif to bacteria.

1 33. The method according to Claim 29, 30, 31, or 32 in  
2 which the carbohydrate is selected from the group  
3 consisting of forssman glycolipid and globoside.

1 34. The method according to Claim 30 or 32 in which the  
2 second carbohydrate is selected from the group consisting  
3 of asialo-GM1 and asialo-GM2.

1 35. The method according to Claim 31 or 32 in which the  
2 third carbohydrate is N-acetyl-D-glucosamine.

1 36. The method according to Claim 29, 30, 31, or 32 in  
2 which the carbohydrate or carbohydrates are multivalent.

1 37. The method according to Claim 29, 30, 31, or 32 in  
2 which at least one carbohydrate is detectably labeled.

1 38. The method according to Claim 29, 30, 31, or 32 in  
2 which at least one carbohydrate is attached to a solid  
3 phase support.

1 39. A kit for detecting the presence of S. pneumoniae in  
2 a sample from a subject comprising:

- 3 (a) a carbohydrate containing a disaccharide N-  
4 acetyl-D-galactosamine B1-3Gal motif; and  
5 (b) means for detecting binding of the carbohydrate  
6 to a bacterium in a sample from the subject.

1 40. The kit of Claim 39, further comprising:

- 2 (c) a second carbohydrate containing a disaccharide  
3 N-acetyl-D-galactosamine B1-4Gal motif; and  
4 (d) means for detecting binding of the second  
5 carbohydrate to the bacterium.

- 1 41. The kit of Claim 39, further comprising:  
2 (e) a third carbohydrate containing a disaccharide  
3 N-acetyl-D-glucosamine motif; and  
4 (f) means for detecting binding of the third  
5 carbohydrate to the bacterium.
- 1 42. The kit of Claim 40, further comprising:  
2 (e) a third carbohydrate containing a disaccharide  
3 N-acetyl-D-glucosamine motif; and  
4 (f) means for detecting binding of the third  
5 carbohydrate to the bacterium.
- 1 43. The kit of Claim 39, 40, 41, or 42 in which at least  
2 one carbohydrate is detectably labelled.
- 1 44. The kit of Claim 39, 40, 41, or 42 in which at least  
2 one carbohydrate is attached to a solid phase support.

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FIG. 1A

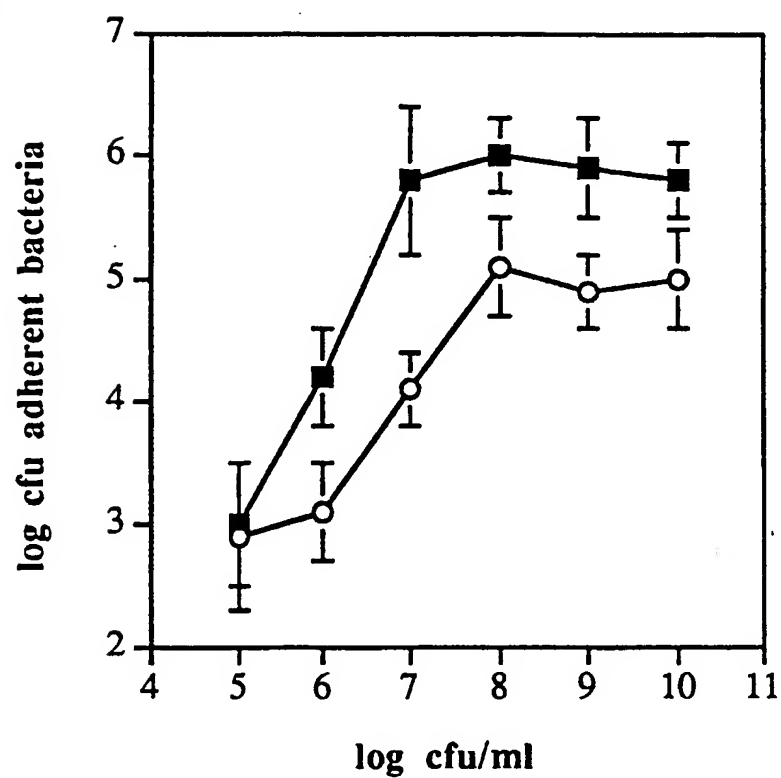
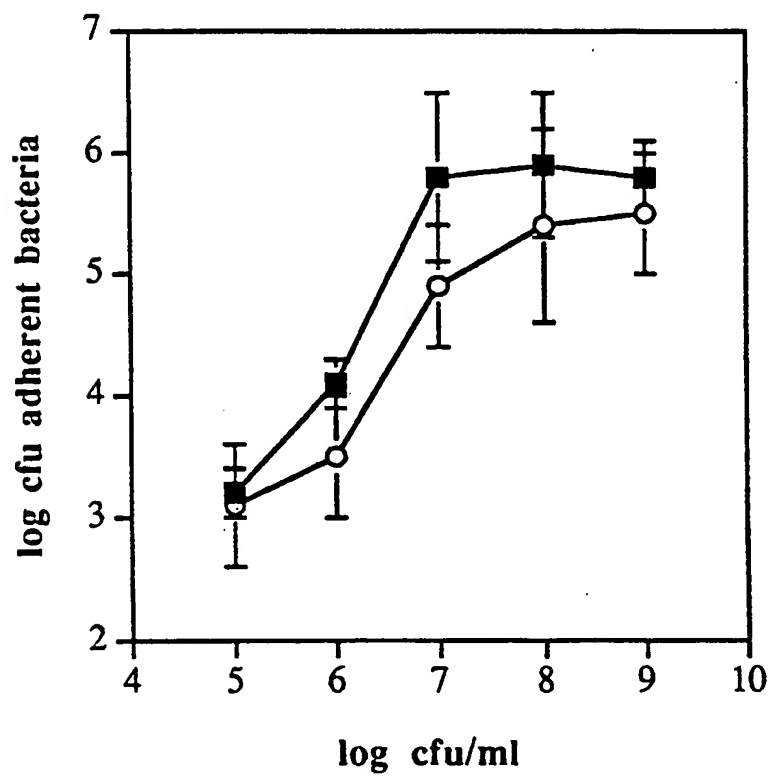


FIG. 1B



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FIG. 2A

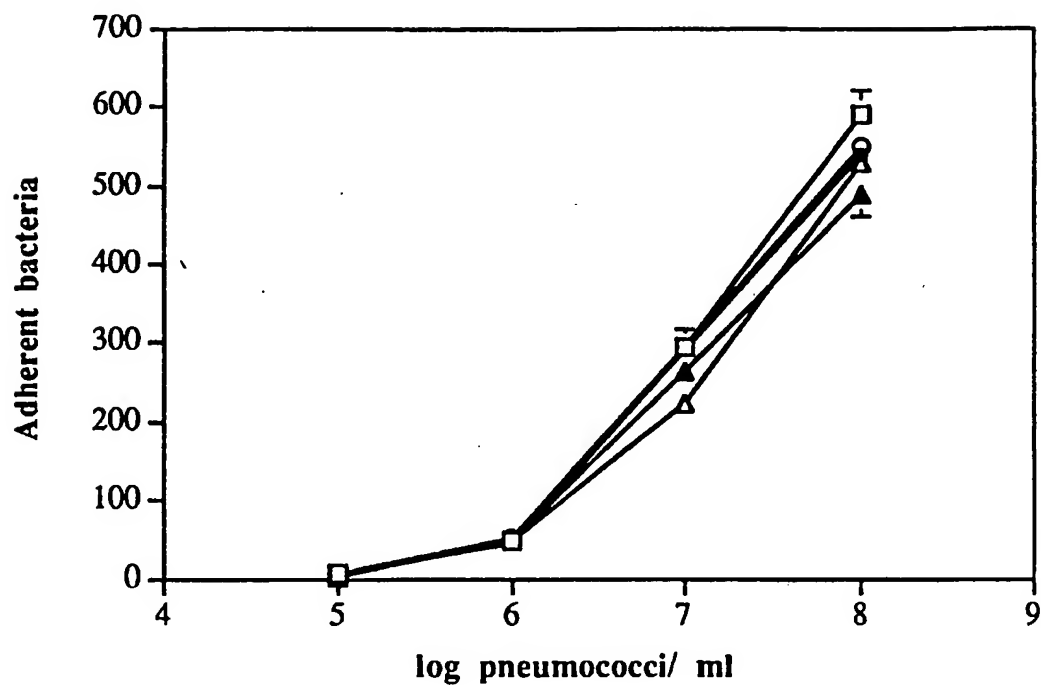
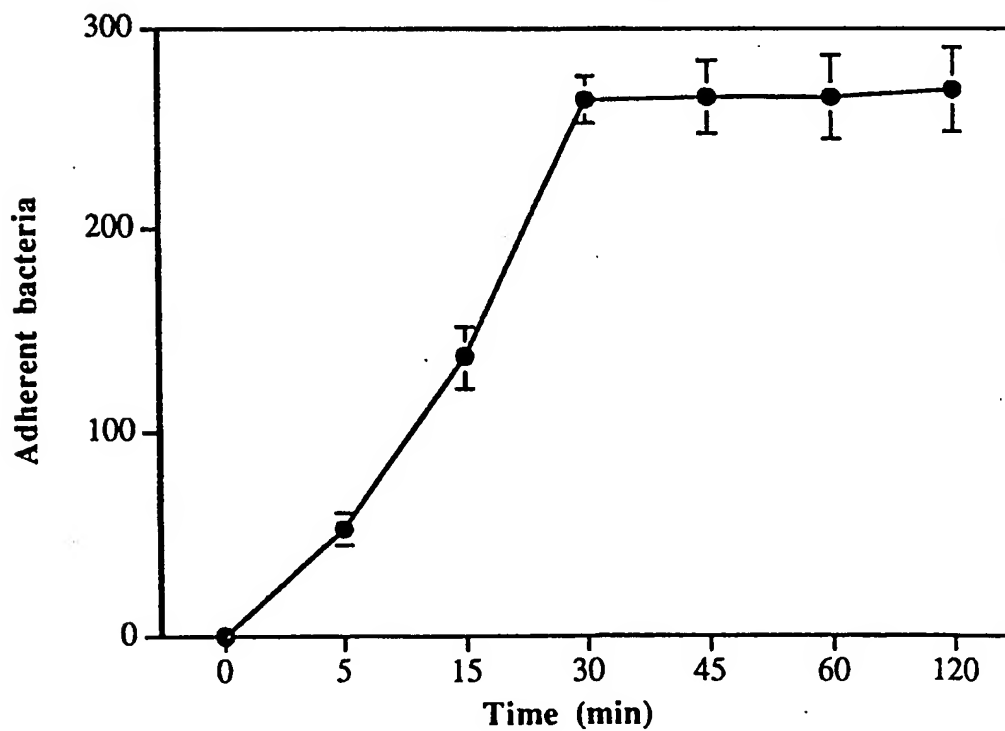


FIG. 2B



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FIG. 3A

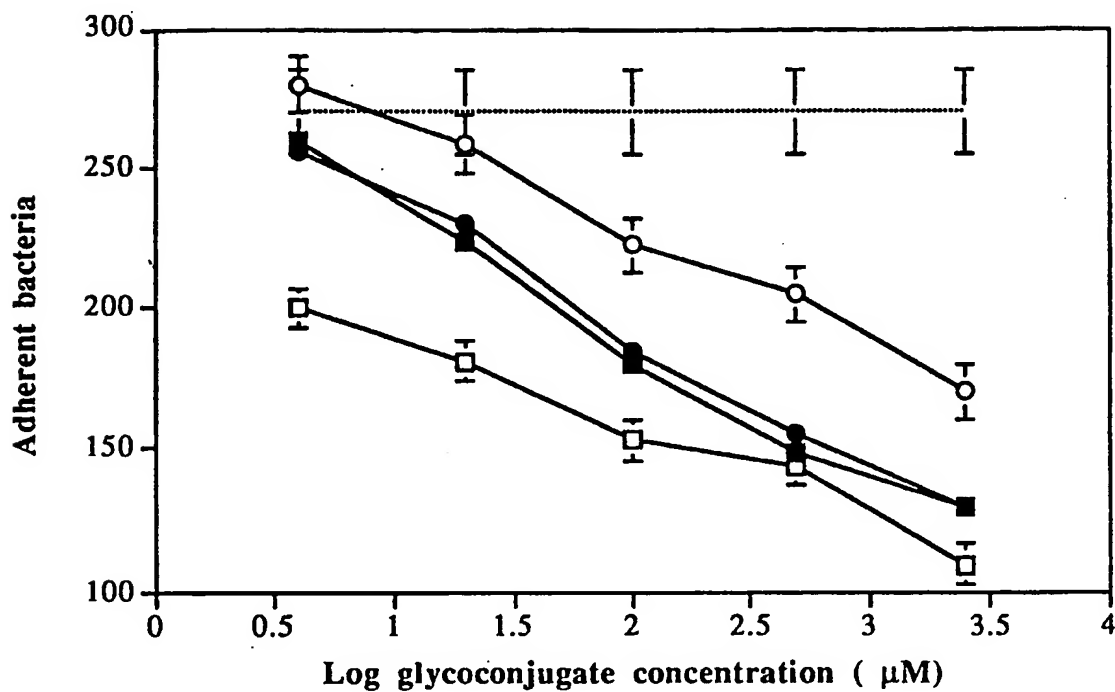
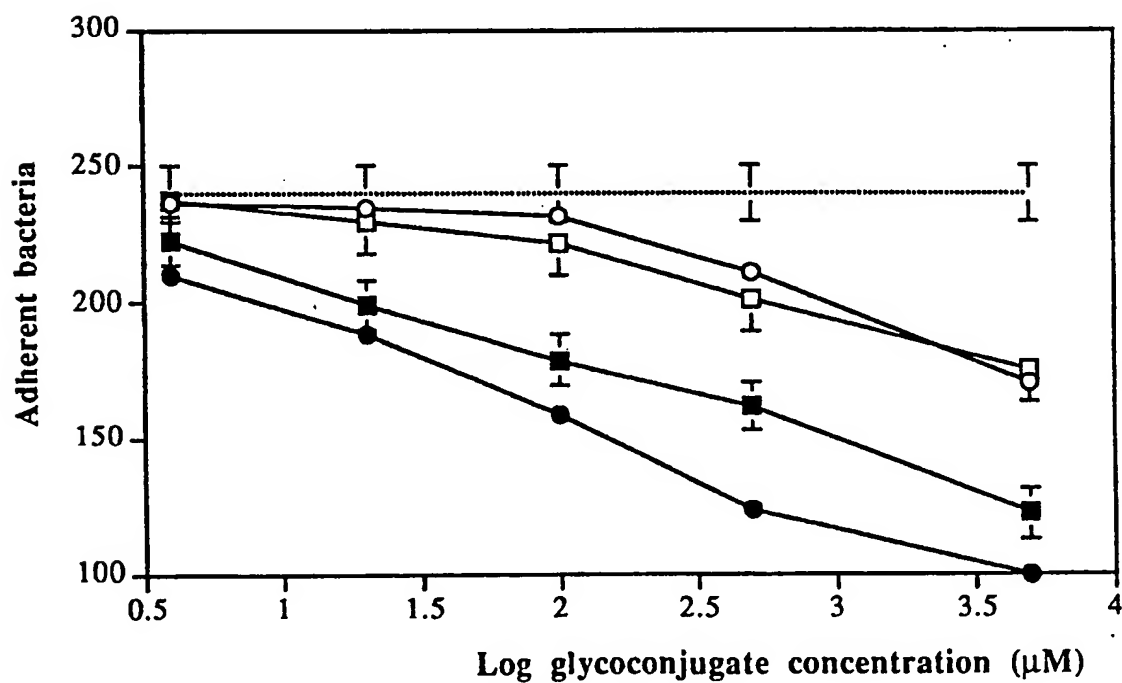
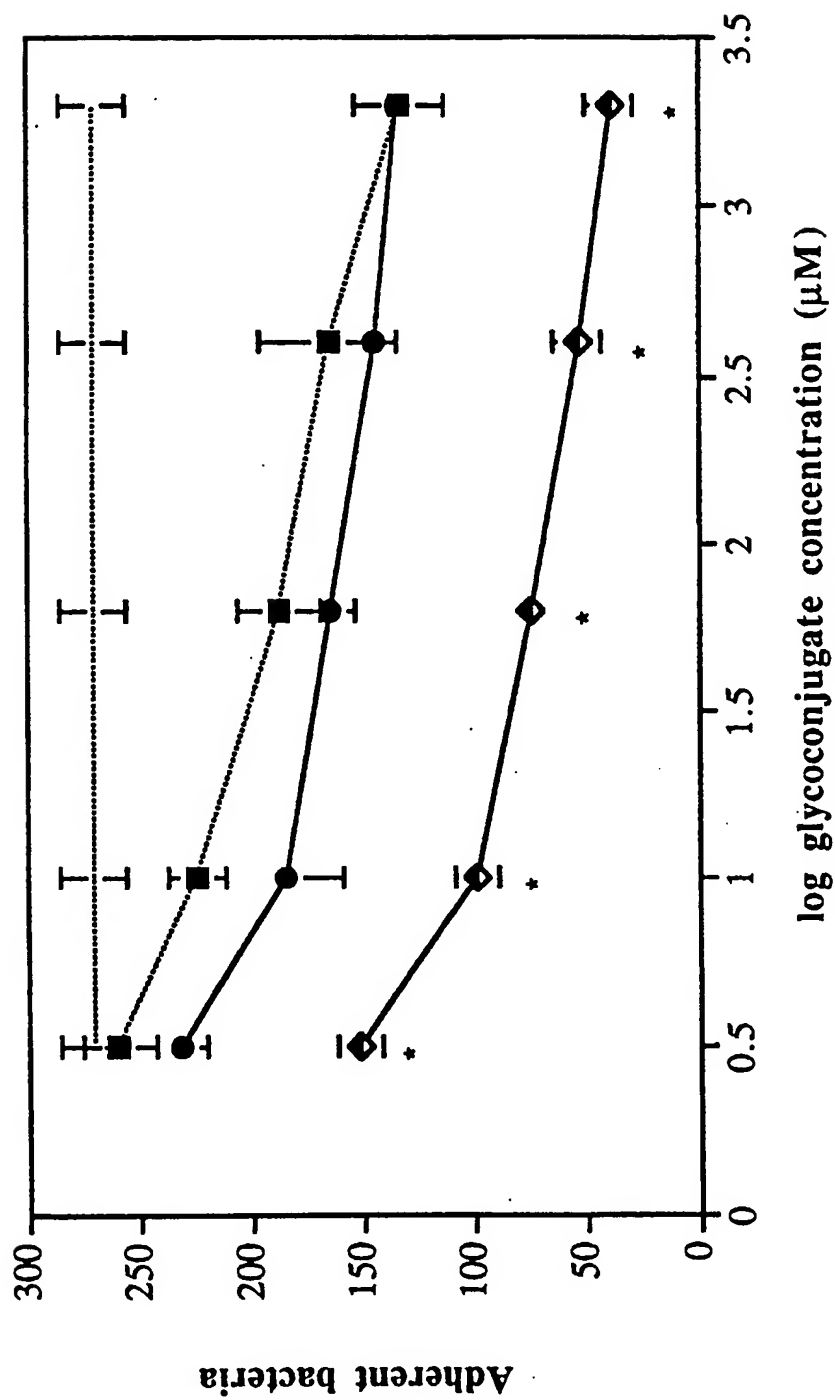


FIG. 3B



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FIG. 4A





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FIG. 4B

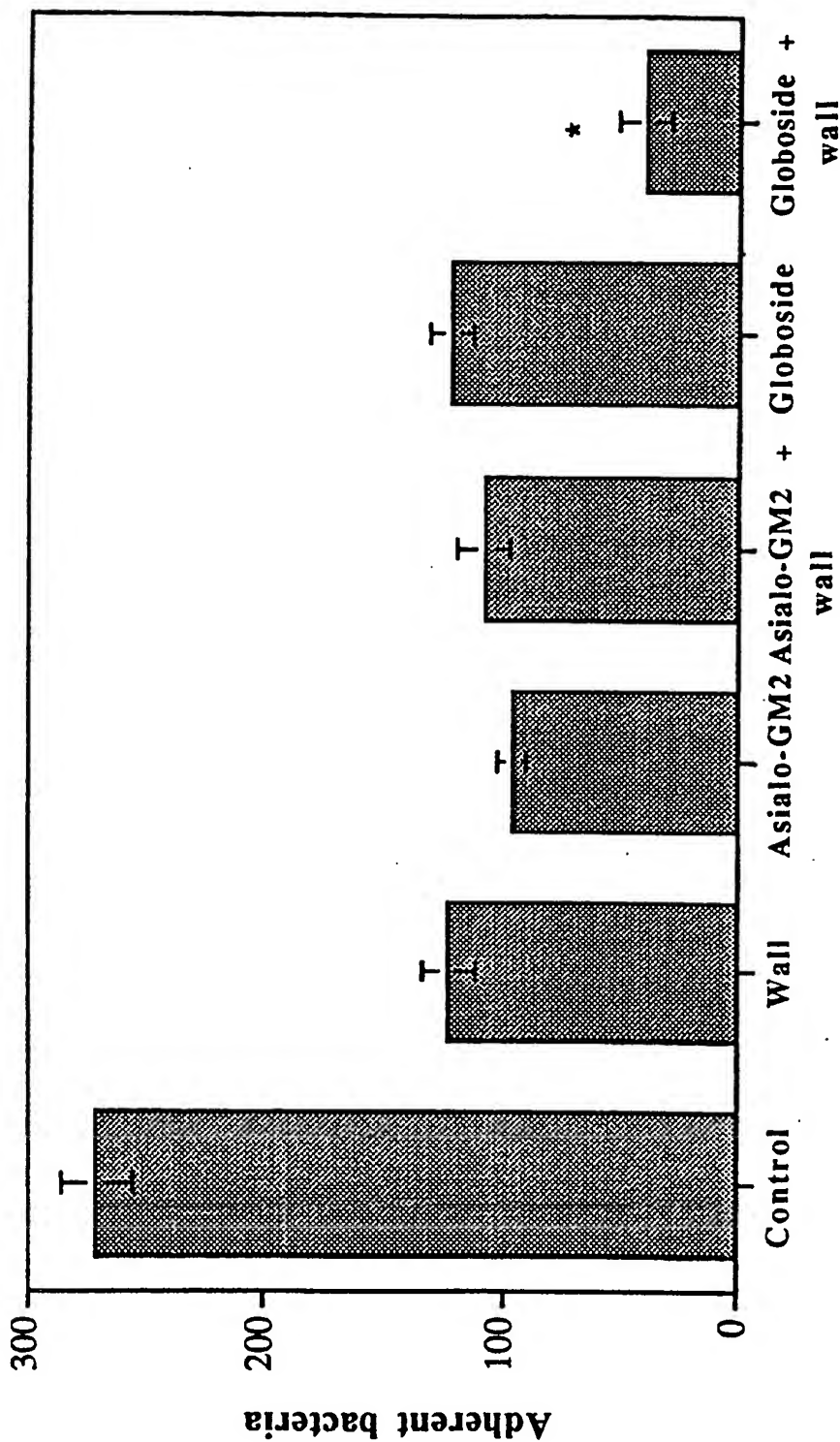


FIG. 5A

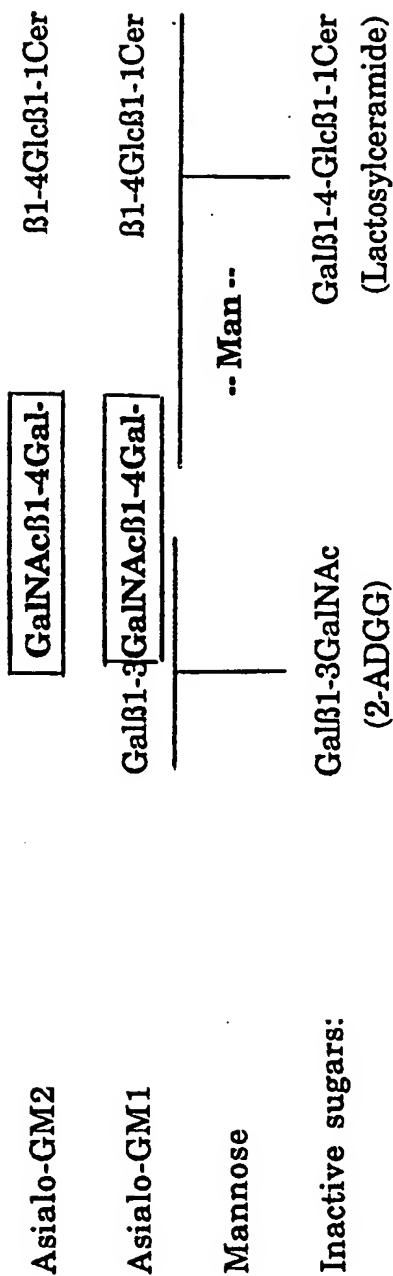


FIG. 5B

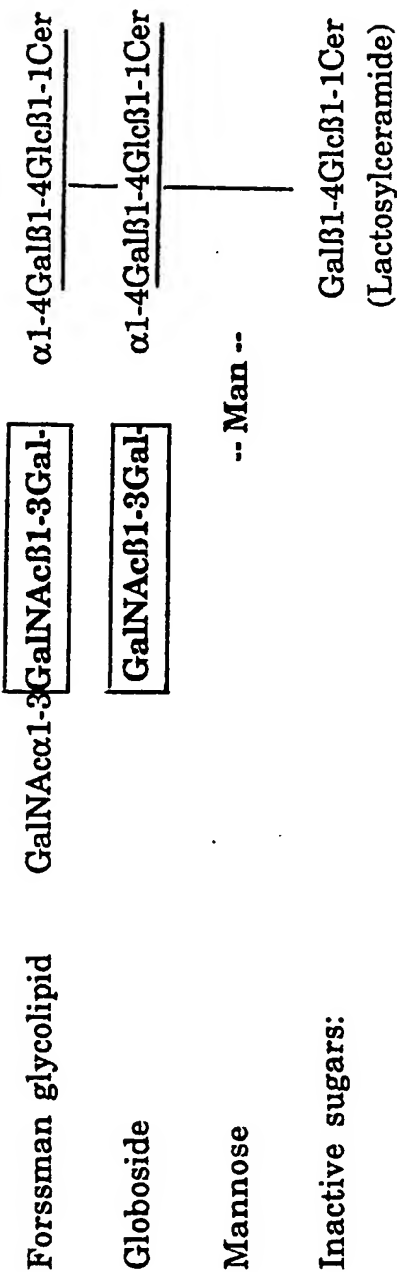


FIG. 6A

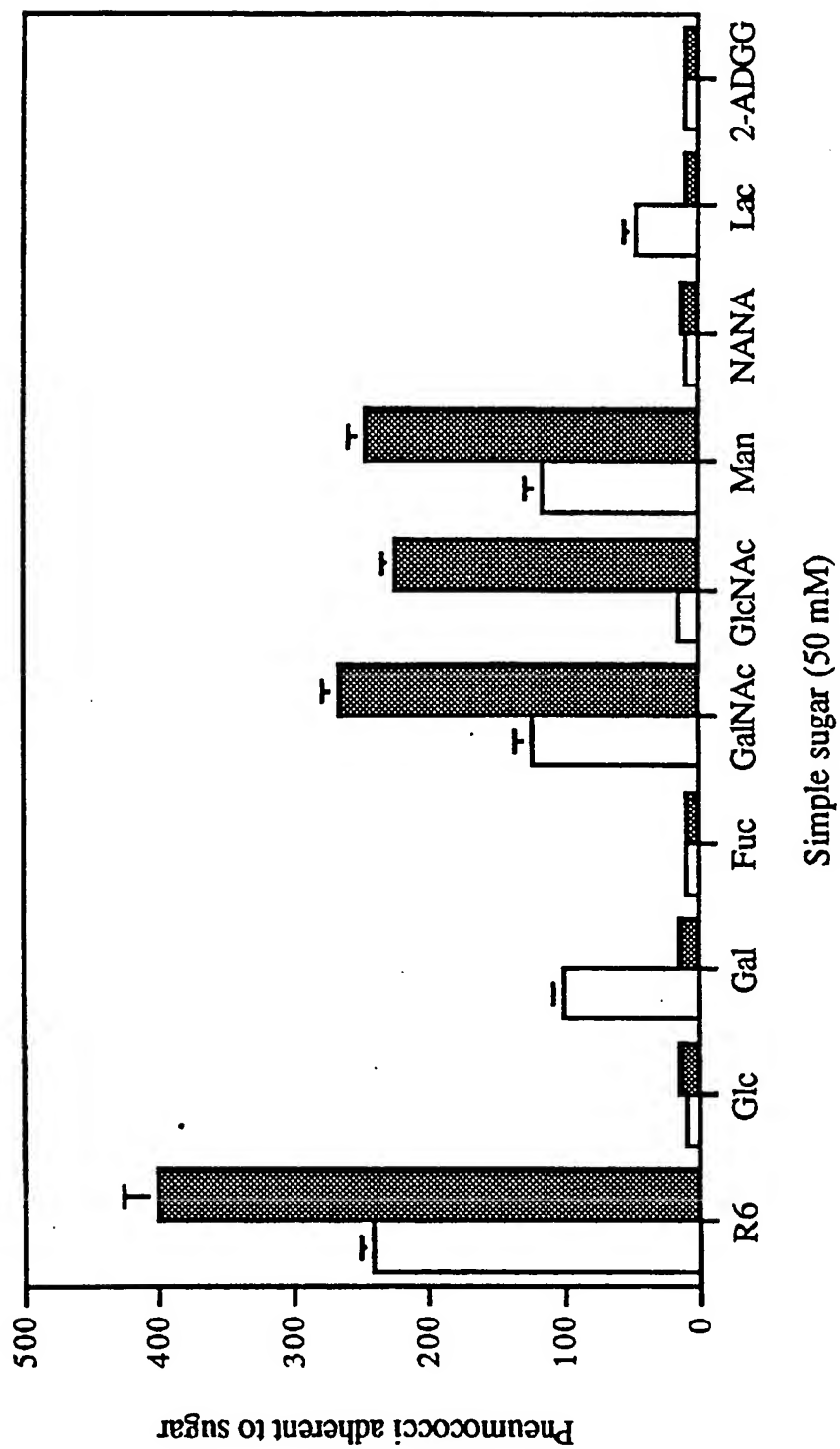
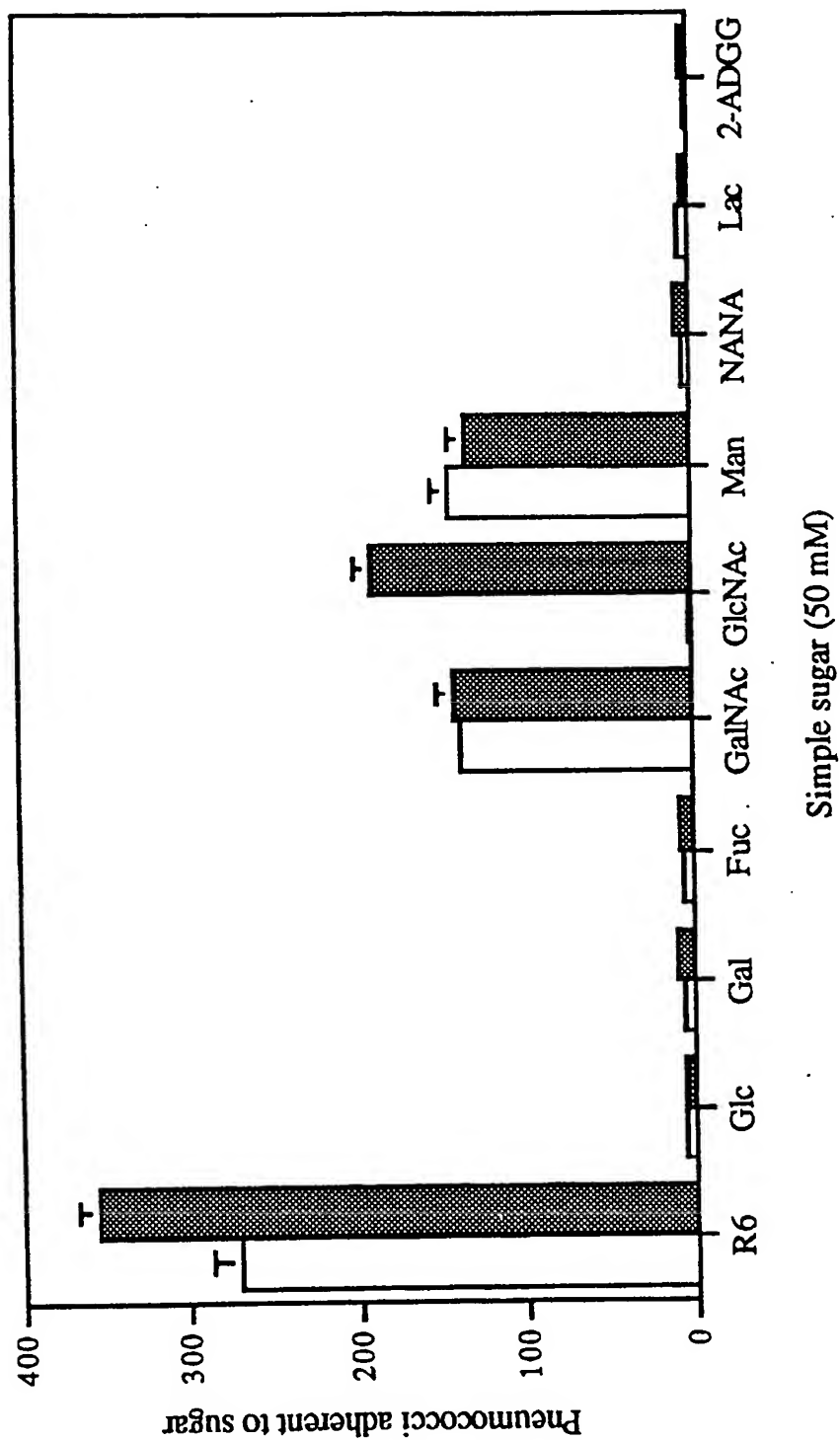
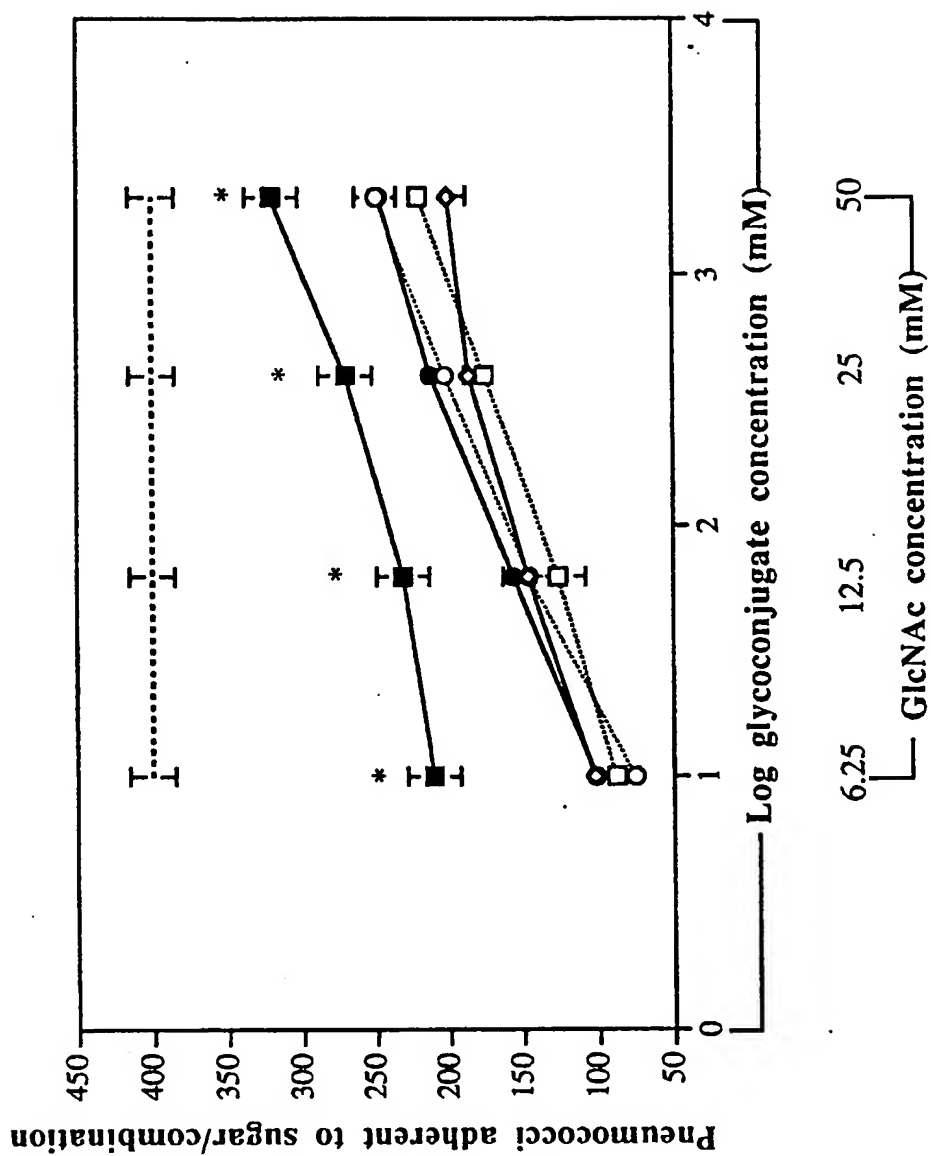


FIG. 6B



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FIG. 7



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 31/70, 31/55, 31/34, G01N 33/569</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 95/33467</b> <b>(43) International Publication Date:</b> 14 December 1995 (14.12.95)
<b>(21) International Application Number:</b> PCT/US95/07209 <b>(22) International Filing Date:</b> 6 June 1995 (06.06.95) <b>(30) Priority Data:</b> 08/254,577 6 June 1994 (06.06.94) US <b>(71) Applicant:</b> THE ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021-6399 (US). <b>(72) Inventors:</b> TUOMANEN, Elaine, I.; Apartment 12C, 430 East 63rd Street, New York, NY 10021 (US). CUNDELL, Diana, R.; Apartment 5K, 430 East 63rd Street, New York, NY 10021 (US). <b>(74) Agents:</b> FEHLNER, Paul, F. et al.; Klauber & Jackson, Continental Plaza, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).	<b>(81) Designated States:</b> AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, IS, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SK, TM, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 4 January 1996 (04.01.96)	
<b>(54) Title:</b> MODULATORS OF PNEUMOCOCCAL ADHERENCE TO PULMONARY AND VASCULAR CELLS AND DIAGNOSTIC AND THERAPEUTIC APPLICATIONS		
<b>(57) Abstract</b>  The present invention relates to compositions and methods for preventing pneumococcal infection. In particular, this invention relates to the identification of the minimum receptor targets of pneumococcal adherence to pulmonary and vascular endothelium, and to compositions and methods for preventing such adherence. In particular, the invention relates to the ability of one or more carbohydrate entities having the following motif or motifs: a disaccharide N-acetyl-D-galactosamine $\beta$ 1-3Gal motif, a disaccharide N-acetyl-D-galactosamine $\beta$ 1-4Gal motif, and an N-acetyl-D-glucosamine motif, effective to induce elution of adherent <i>S. pneumoniae</i> from host cells. In particular, a composition containing all three motifs can elute about 100 % of pneumococcal bacteria from lung epithelial cells, and from venous endothelial cells. In a particular embodiment, a pharmaceutical composition of the invention can be used to treat pneumococcal infections in which the host cells are lung epithelial cells. For use in blocking adherence to, or eluting adherent bacteria from, lung epithelial cells, the pharmaceutical composition is an aerosol formulation. The invention further provides formulations for parenteral administration for treating systemic blood-borne infections by preventing or reversing binding to venous endothelial cells. The invention further relates to methods and kits for determining the presence of pneumococci in a biological sample from a subject.		

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/07209

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K31/70 A61K31/55 A61K31/34 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	INFECT. IMMUN., 1995, 757-61, CUNDELL, DIANA R. ET AL 'Relationship between colonial morphology and adherence of Streptococcus pneumoniae' see the whole document ---	1-44
P, X	MICROB PATHOG, DEC 1994, 17 (6) P361-74, ENGLAND, CUNDELL DR ET AL 'Receptor specificity of adherence of Streptococcus pneumoniae to human type-II pneumocytes and vascular endothelial cells in vitro.' see the whole document especially abstract & page 365, table 1 --- -/--	1-44

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

14 November 1995

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/US 95/07209

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MICROBIAL PATHOGENESIS, vol. 17, no. 1, July 1994 pages 63-68, SUNDBERG-KÖVAMEES, M. ET AL 'Specific binding of Streptococcus pneumoniae to two receptor saccharide structures' see the whole document ---	2,6,9, 18,22, 30,34,40
A	PROCEEDINGS NATIONAL ACADEMY SCIENCES, USA, vol. 85, August 1988 pages 6157-6161, KIRVAN, H.C. ETAL 'Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcB1-4Gal found in some glycolipids' cited in the application see the whole document ---	1-44
X	OTOLARYNGOLOGY-HEAD AND NECK SURGERY, vol. 92, no. 3, 1984 pages 266-269, ANDERSSON, B. ET AL 'Attachment of Streptococcus pneumoniae to human pharyngeal epithelial cells in vitro-mechanism of binding' see page 267, column 2; table 3 see page 268, column 1, line 12 see page 269, column 1, line 35 - column 2, line 5 ---	1,5,16, 17,21
X	US,A,5 217 715 (KRIVAN ET AL) 8 June 1993  see the whole document especially column 2, line 1-43 ---	2,6,9, 16,18, 22,25, 30,34, 36,38, 40,44
X	WO,A,91 16449 (UNIV PENNSYLVANIA) 31 October 1991 cited in the application see the whole document especially page 24, line 23-30 & claim 28 --- -/--	1,2, 9-18, 25-28

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/07209

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ZENTRALBL BAKTERIOL MIKROBIOL HYG [A], JUN 1987, 265 (1-2) P160-8, GERMANY, WEST, BEUTH J ET AL 'Lectin mediated adhesion of Streptococcus pneumoniae and its specific inhibition in vitro and in vivo.'</p> <p>see the whole document especially abstract; page 162, line 7-12; page 167</p> <p>---</p>	<p>3,4,7, 10, 14-16, 19,20, 23,31, 32,35, 41,42</p>
A	<p>JOURNAL OF EXPERIMENTAL MEDICINE, vol. 158, August 1983 pages 559-570, ANDERSSON, B. ET AL 'Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells' cited in the application see the whole document</p> <p>---</p>	<p>1-44</p>
A	<p>WO,A,94 03184 (NEOSE PHARMACEUTICALS INC) 17 February 1994 cited in the application see the whole document</p> <p>-----</p>	<p>1-44</p>

# INTERNATIONAL SEARCH REPORT

Int. lation application No.

PCT/US 95/07209

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-44  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
In view of the large number of compounds which are theoretically defined by claims 1-3 the search had to be restricted on economic grounds to those compounds specifically claimed and the general idea of the application.
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/07209

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5217715	08-06-93	US-A- 5389521	14-02-95
		US-A- 5386027	31-01-95
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		IN-A- 173130	12-02-94
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		QA-A- 9678	15-05-93
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